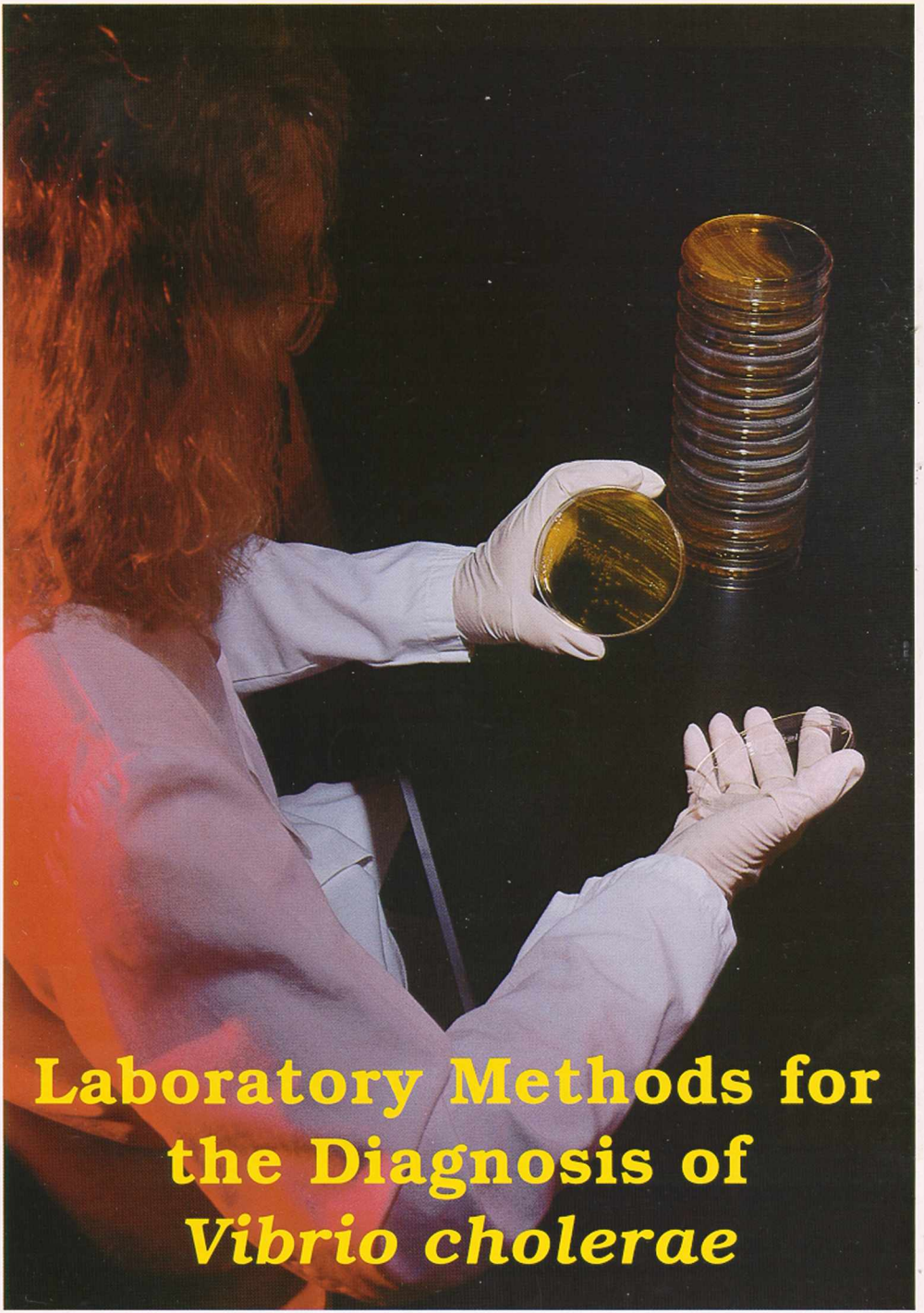

Laboratory Methods for the Diagnosis of *Vibrio cholerae*

CDC/NCID
CENTERS FOR DISEASE CONTROL AND PREVENTION
NATIONAL CENTER FOR INFECTIOUS DISEASES

PRO SALUTE NOVI MUNDI

This manual was prepared by the National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, in cooperation with the Pan American Health Organization (PAHO), Washington, D.C., USA.

David Satcher, M.D., Ph.D., Director, CDC

James M. Hughes, M.D., Director, National Center for Infectious Diseases (NCID)

Mitchell L. Cohen, M.D., Director, Division of Bacterial and Mycotic Diseases, NCID

Carlyle Guerra de Macedo, M.D., M.P.H., Director, PAHO, Regional Office for the Americas of the World Health Organization

David Brandling-Bennett, M.D., Director, Division of Communicable Disease Prevention and Control

José María Paganini, M.D., Dr.P.H., Director, Division of Health Systems and Services

Virgilio Escutia, Dr.P.H., Regional Advisor in Laboratory Services

Production

Publications Activity, Office of Program Resources, National Center for Infectious Diseases, CDC

Polyxeni M. Potter, Lynne McIntyre, Beverly Holland

Photos by

James D. Gathany

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Introduction

Cholera is a new and reemerging public health concern in many parts of the world. The appearance of epidemic cholera in Latin America and the emergence in Asia of disease caused by the newly identified strain *Vibrio cholerae* O139 indicate that cholera will remain a challenge for years to come. Meeting this challenge with effective cholera prevention and control measures will require application of reliable microbiological techniques.

This manual summarizes laboratory techniques used in the diagnosis of cholera; it will provide a reference for laboratory personnel who establish diagnostic or surveillance techniques for *Vibrio cholerae*. The procedures described are not new; most have been used for a number of years. Even though they require a spectrum of laboratory capabilities, these procedures were selected because of their utility, ease of performance, and ability to give reproducible results. The diversity of laboratory facilities and the availability of materials and supplies were also taken into account. Experimental or research-oriented methods were intentionally not included.

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The following persons are gratefully acknowledged for their valuable contributions toward the production of this manual:

Coordinators

Cheryl A. Bopp, Bradford A. Kay, Joy G. Wells, National Center for Infectious Diseases, CDC

Contributors

Carolyn N. Baker, Timothy J. Barrett, Paul A. Blake, Frances W. Brenner, Lois E. Britt, Daniel N. Cameron, J. J. Farmer, III, John C. Feeley, Patricia I. Fields, James H. Green, Fay B. Hendricks, Katherine D. Greene, Tanja Popovic, Nancy D. Puhr, Nancy A. Strockbine, Robert V. Tauxe, Fred C. Tenover, Jessica Tuttle, I. Kaye Wachsmuth, National Center for Infectious Diseases; Theo M. Hawkins, Public Health Practice Program Office, CDC

Edwin F. Archbold Sanjur, Pan American Health Organization, Washington, D.C.; Elizabeth Castaneda del Gordo, National Institute of Health, Bogota, Colombia; Jose Ramiro Cruz, Institute of Nutrition of Central America, Guatemala City, Guatemala; Alfredo Davila Araujo, National Institute of Hygiene and Tropical Medicine, Guayaquil, Ecuador; Angelo DePaola, Jr., Food and Drug Administration, Dauphin Island, Alabama, USA; Elisa L. Elliot, Food and Drug Administration, Washington, D.C., USA; Silvia Giono Cerezo, Institute of Reference Diagnostics and Epidemiology, Mexico City, Mexico; Walter E. Hill, Food and Drug Administration, Bothell, Washington, USA; Anwarul Huq, University of Maryland, College Park, Maryland, USA; Charles A. Kaysner, Food and Drug Administration, Bothell, Washington, USA; Miguel Kourany, Ministry of Health, Panama City, Panama; Nathaniel F. Pierce, World Health Organization, Geneva, Switzerland; R. Bradley Sack, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh; Elsa Sofia Toro Araujo, National Institute of Hygiene "Rafael Rangel," Caracas, Venezuela

International Advisory Group

Gladys Estacio Addimandi, National Institute of Hygiene "Rafael Rangel," Caracas, Venezuela; Norma Binsztein, National Institute of Microbiology "C.G. Malbran," Buenos Aires, Argentina; Erika Hannover Habetswallner, National Institute of Health, La Paz, Bolivia; Kinue Irino,

Institute "Adolfo Lutz," Sao Paulo, Brazil; Maria Amelia Flores Gonzalez, Ministry of Public Health, Guatemala City, Guatemala; Miguel Francisco Torres Ruben, Guatemalan Social Security Institute, Guatemala City, Guatemala; Anna Patricia Velez Moller, San Carlos University, Guatemala City, Guatemala; Luis Rodrigo Mora Fonseca, Costa Rican Social Security Laboratory, San Jose, Costa Rica; Zandra E. Fuentes Jimenez, Ministry of Health, San Salvador, El Salvador; Luis Eduardo Julio Villaverde, Central Health Laboratory, Panama City, Panama; Raul Joaquin Monte Boada, Institute of Tropical Medicine "Pedro Kouri," Havana, Cuba; Wally Silva San Cristobal, Institute of Public Health, Santiago, Chile; Guadalupe Perez Rueda, National Institute of Hygiene, Quito, Ecuador; Carlos Carrillo Parodi, Peruvian University, Cayetano Heredia, Lima, Peru; Milagros Peralta, Dominican Institute of Industrial Technology, Santo Domingo, Dominican Republic; Isabel Gonzalez Gonzalez, Central Public Health Laboratory, Montevideo, Uruguay; Zaida Josefina Carvajal Tesorero, Biomedical Institute, Caracas, Venezuela; Coromoto Sandoval De Diaz, Ministry of Health, Caracas, Venezuela; Yecenia Lopez de Beauperthuy, National Institute of Hygiene "Rafael Rangel," Caracas, Venezuela

Authors' Note

Toxigenic *V. cholerae* O139, A Newly Recognized Cause of Cholera

Before 1992, of the more than 130 serogroups of *Vibrio cholerae* that have been reported, only the O1 serogroup was associated with epidemic and pandemic cholera. Members of the so-called "non-O1" serogroups had been associated only with extraintestinal infections and with sporadic cases and occasional limited outbreaks of diarrhea and were not considered to be of major public health importance.

This is the situation described in the text of this manual. In late 1992 and early 1993, after the text of this manual was complete, large outbreaks of cholera due to non-O1 *V. cholerae* were first reported in India and Bangladesh. The agent responsible for these outbreaks was identified as a strain of *V. cholerae* belonging to a newly described serogroup, O139. This strain produced cholera toxin but not the heat-stable enterotoxin sometimes associated with non-O1 *V. cholerae* strains. Most of the O139 isolates were reported to be resistant to trimethoprim-sulfamethoxazole, streptomycin, furazolidone, and the vibriostatic agent O/129.

As of this writing, the epidemiologic characteristics of the O139 serogroup appear to be similar to those of the O1 serogroup. The importance of the O139 serogroup and its relationship to the O1 serogroup have not yet been determined and will undoubtedly be elaborated within the coming years. The cultural and biochemical characteristics of the O139 serogroup are identical to those of *V. cholerae* O1, as well as to those of all other serogroups of *V. cholerae*. Accordingly, until specific information is available, the isolation and biochemical identification procedures in this manual for the O1 serogroup should apply as well to the O139 serogroup, except that O139 antiserum is required for identification. Biotyping tests for *V. cholerae* O1 are not valid for *V. cholerae* O139 or any non-O1 serogroup isolates. Specific O139 antiserum is being produced and is likely to be available commercially. Throughout this manual, the use of the term "non-O1" should be understood to refer to all serogroups other than O1, except for the recently described O139 serogroup.

The circumstances under which laboratory diagnosis of cholera is most helpful and the recommendations for when stool cultures should be obtained are not changed by the appearance of this new serogroup of *V. cholerae*. However, it makes the public health evaluation of non-O1 *V. cholerae* isolates more complex. Because non-O1 strains (other than O139) are common in many parts of the world, the approach to this evaluation depends on the frequency with which one expects to find O139 strains. This means that the best approach varies with the setting and will change if this strain persists and becomes more widespread. We recommend the following:

In areas not known to have *V. cholerae* O139 infections

Sporadic isolates of non-O1 *V. cholerae* need not be characterized further for public health purposes unless an epidemiologic link exists with a part of the world known to be affected by O139 (at this writing, India and Bangladesh) or the disease is typical of severe cholera, with life-threatening dehydration. When further characterization is indicated, it should include agglutination in O139 antiserum and testing for production of cholera toxin.

Isolates of non-O1 *V. cholerae* from outbreaks (i.e., more than one linked case) of cholera-like illness should be tested for agglutination in O139 antiserum and for production of cholera toxin. Strains that produce cholera toxin but are not of serogroup O1 or O139 should be sent to a reference laboratory for determination of the serogroup.

In areas known to have *V. cholerae* O139 infections

Sporadic isolates of non-O1 *V. cholerae* should be tested for agglutination in O139 antiserum.

Isolates of non-O1 *V. cholerae* from outbreaks of cholera-like illness should be tested for agglutination in O139 antiserum. If the isolates are not of serogroup O139, they should be tested for production of cholera toxin. Strains that produce cholera toxin but are not of serogroup O1 or O139 should be sent to a reference laboratory for determination of the serogroup.

We also recommend that infection with toxigenic *V. cholerae* O139 should be handled and reported in the same manner as that caused by toxigenic *V. cholerae* O1. The associated diarrheal illness should be called cholera and should be reported as a case of cholera to the appropriate public health authorities.

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I. Etiology and Epidemiology of Cholera

Epidemic cholera is caused by the O1 serogroup of *Vibrio cholerae*. This serogroup is one of over 130 *V. cholerae* serogroups that have been identified but is the only one associated with epidemic or pandemic cholera. Because the pathogenic potential of *V. cholerae* isolates varies considerably, the laboratory and epidemiologic distinctions between O1 and non-O1 *V. cholerae* isolates are of paramount importance. Isolates of non-O1 *V. cholerae* can cause illness because they can possess a variety of virulence factors, including production of cholera toxin (CT). However, they do not pose the public health threat of the O1 serogroup. Non-O1 *V.cholerae* isolates should be reported in a way that avoids confusion with organisms of the O1 serogroup. (See Authors' Note for a discussion of *V. cholerae* O139.)

Within the O1 serogroup, the ability to produce CT is a major determinant of virulence. In general, the isolates of *V. cholerae* of the O1 serogroup that produce CT are considered fully virulent and fully capable of causing epidemic cholera (Figure I-1). Most *V. cholerae* isolated during cholera outbreaks will be toxigenic serogroup O1. However, some isolates of *V. cholerae* O1 do not produce CT and cannot cause epidemic cholera. When these isolates are encountered, they must be considered within their clinical and epidemiologic context. Like non-O1 isolates, these isolates may be associated with sporadic diarrheal disease.

Isolates of *V. cholerae* O1 can be divided into two biotypes, El Tor and classical, on the basis of several phenotypic characteristics (see Chapter VI, "Laboratory Identification of *V. cholerae*" for a discussion of biotyping methods). Cholera pandemics before 1960 were caused by *V. cholerae* O1

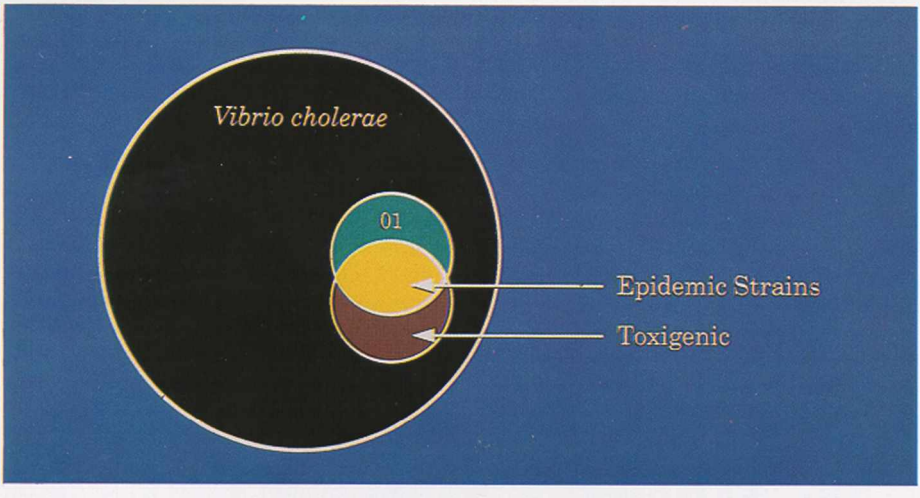

Figure I-1. Of strains of *V. cholerae*, only toxigenic members of serogroup O1 have traditionally had epidemic potential and importance as agents of cholera.

belonging to the classical biotype. The pandemic which began in 1961 (known as the seventh pandemic) was associated with the El Tor biotype. Currently the El Tor biotype is responsible for virtually all of the cholera cases throughout the world, and classical isolates are not encountered outside Bangladesh.

A. Historical Background—Pandemic Cholera

Cholera is thought to have its ancestral home in the Ganges Delta of the Indian subcontinent. In the nineteenth century, pandemic waves of cholera spread to many parts of the world from southern Asia along routes of trade, pilgrimage, and migration. During these pandemics, large urban epidemics with high mortality rates occurred throughout Europe and the Americas. By 1860, investigations by John Snow and others showed that water supplies contaminated with sewage were the principal route of transmission. As a result, well before the causative agent was identified, fear of recurrent epidemic cholera brought about the "sanitary reform" movement, which vastly improved water systems and methods of sewage disposal in the industrialized world.

Because cholera was often introduced into an area from other affected areas by ship, surveillance and disease reporting became important. The threat of epidemic cholera led to routine disease notification, and public health departments were created to investigate suspect cases. By the 1880s, because of the efforts of public health agencies, epidemic spread was prevented when cholera patients from countries affected by the fifth pandemic (1881-1896) disembarked in North American ports. Since that time, safe drinking water and hygienic treatment of sewage have been credited with protecting many populations from epidemic cholera and other infectious diseases. By the middle of the twentieth century, cholera was restricted to a few countries in Asia.

The relatively limited geographic range of cholera in the 1950s was greatly expanded in the beginning of the next decade. In 1961, a massive epidemic began in Southeast Asia. This epidemic is now recognized as the beginning of the seventh cholera pandemic. This pandemic was caused by the El Tor biotype of toxigenic *V. cholerae* O1. It spread rapidly through south Asia, the Middle East, and southeastern Europe, reaching Africa by 1970. Several outbreaks occurred in Europe as a result of contaminated shellfish beds or untreated water supplies. Until 1991, the Americas were relatively unaffected by the seventh pandemic (Figure I-2), although a few imported cases occurred annually in many industrialized countries.

In January 1991, epidemic cholera appeared in South America in several coastal cities of Peru and spread rapidly to adjoining countries. By the end of 1991, cholera had spread to 18 countries in Latin America, with over 391,000 cases and nearly 3900 deaths. By early 1993, it had spread to all but one Latin American country. There is no evidence that cholera will soon disappear from Latin America.

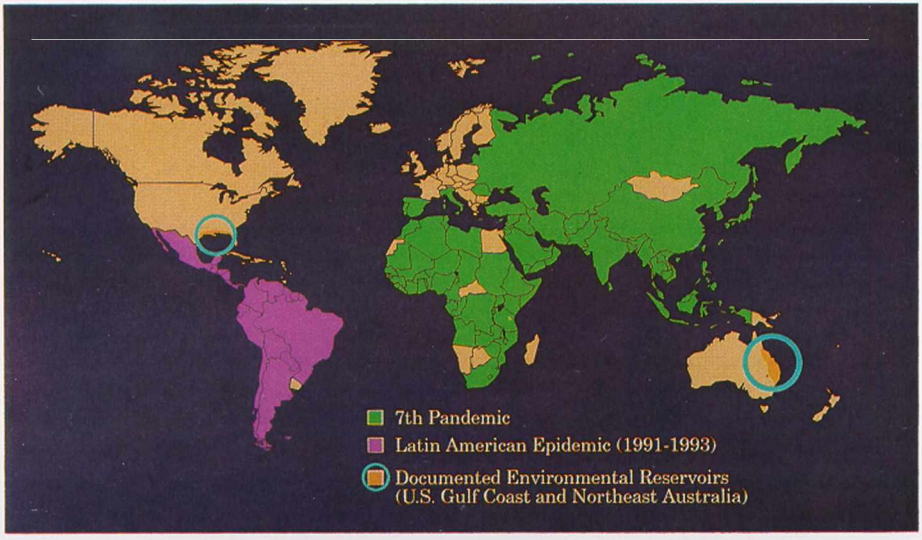

Figure I-2. Beginning in 1961, cholera spread rapidly from Indonesia in what is now known as the seventh cholera pandemic (green shading), and by the early 1970s had reached Africa and Europe. In January of 1991, it spread to the Western Hemisphere and was rapidly disseminated throughout Latin America (red shading). Environmental reservoirs of unrelated toxigenic organisms are located in northeast Australia and the U.S. Gulf Coast (blue circles). (Based on cases reported to the World Health Organization, excluding imported cases)

B. Environmental Foci

Because cholera has historically appeared in epidemic waves, it was thought that no permanent natural reservoirs existed in most of the world. However, in recent years, foci of endemic toxigenic *V. cholerae* O1 in the environment have been identified. For example, cholera cases have repeatedly occurred among persons who drank water from remote Australian rivers or ate undercooked crabs from waters of the U.S. Gulf Coast. In both locations, toxigenic *V. cholerae* O1 was isolated from surface waters where contamination with human sewage was unlikely. This indicates that at least some strains of toxigenic *V. cholerae* O1 can persist in the natural environment for many years.

C. Clinical Manifestations

Cholera is a secretory diarrheal disease. The enterotoxin produced by *V. cholerae* O1 causes a massive outpouring of fluid and electrolytes into the bowel. This rapidly leads to profuse watery diarrhea, loss of circulating blood volume, metabolic acidosis, potassium depletion, and ultimately vascular collapse and death. In severe cases, purging diarrhea can rapidly cause the loss of 10% or more of the body's weight, with attendant hypovolemic shock and death; however, 75% or more of initial infections

with *V. cholerae* O1 biotype El Tor may be asymptomatic, depending on the infecting dose. Of the 25% of persons with symptomatic infections, most have mild illness. Approximately 5% of patients have moderate illness that requires medical attention but not hospitalization. In only about 2% of patients does the illness progress to life-threatening "cholera gravis" (Figure I-3). Persons with blood type O are more likely to develop severe cholera than those with other blood types. Because blood type O is more common in Latin America than elsewhere in the world, a greater proportion of cholera infections in this area are likely to be severe.

D. Treatment

Successful treatment of cholera patients depends on rapid replacement of fluid and electrolyte losses. Before the discovery of rehydration therapy, 30% to 50% of patients with severe "cholera gravis" died. Now, with proper treatment, mortality is less than 1% of reported cases. Fluids and electrolytes can be replaced rapidly through either oral or intravenous routes. The oral route is possible because epithelial cells of the bowel absorb fluid and electrolytes when presented with glucose or other carbohydrates, even in the face of active secretion. The optimum mix of electrolytes and glucose for oral treatment of cholera and other secretory diarrheas is used in World Health Organization-approved Oral Rehydration Salts (ORS) solution. Most cholera patients can be treated with ORS solution alone, and even those who require initial intravenous therapy can soon be switched to ORS solution alone. Intravenous therapy is required for patients who are in profound shock or cannot drink. In such

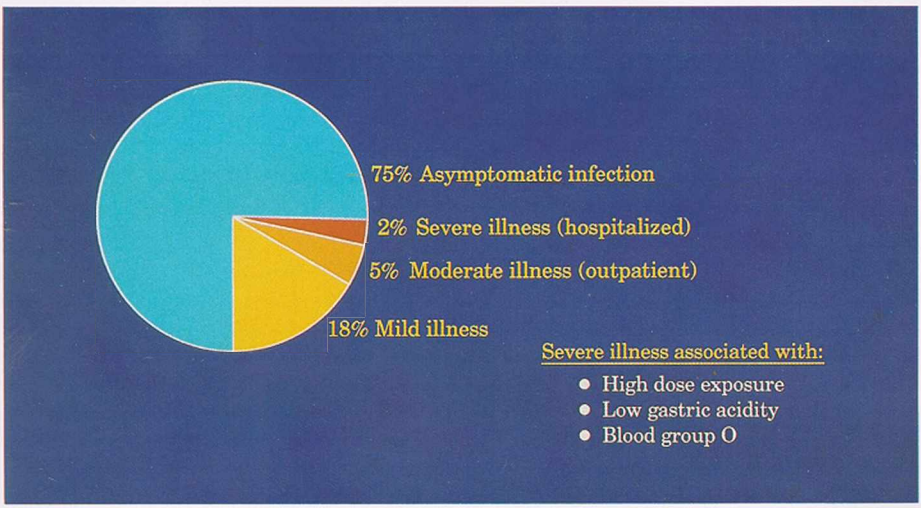

Figure I-3. Most naturally occurring infections with epidemic strains of *V. cholerae* are asymptomatic. Only a small proportion of cases require treatment or are life-threatening. Inoculum dosage and host factors play important roles in the development of disease.

cases, Ringer's lactate solution is recommended for initial intravenous rehydration. Acute volume replacement is also possible with normal or half normal saline, if combined with ORS therapy. Five percent dextrose solution is ineffective and should not be used because it contains no electrolytes.

Antimicrobial therapy is helpful, although not essential, in treating cholera patients. Antimicrobial agents reduce the duration of illness, the volume of stool, and the duration of shedding of vibrios in the feces.

Antimicrobial therapy is sometimes recommended for close contacts of cholera patients. However, prophylactic antimicrobial therapy is not recommended for entire communities because it has proved ineffective, diverts scarce resources, and hastens the appearance of antimicrobial resistance in *V. cholerae* O1. Antimicrobial agents recommended by the World Health Organization for treating cholera patients include tetracycline, doxycycline, furazolidone, trimethoprim-sulfamethoxazole, erythromycin, or chloramphenicol. Recent data suggest that fluoroquinolones (e.g., ciprofloxacin and norfloxacin) are also effective. Because antimicrobial resistance has been a growing problem in some parts of the world, the susceptibility of *V. cholerae* O1 strains to antimicrobial agents should be monitored periodically.

E. Epidemiology

When cholera first appears in epidemic form in an unexposed population, it can affect all age groups. In contrast, in areas with high rates of endemic disease, such as Bangladesh, most of the adult population have gained some degree of natural immunity because of illness or repeated asymptomatic infections. In this setting, the disease occurs primarily in young children, who are exposed to the organism for the first time, and in the elderly, who have lower gastric acid production and waning immunity. Epidemics typically occur in late summer and fall. The poor are at greatest risk because they often lack safe water supplies, have poor hygiene within the home, and may depend on street vendors or other poorly regulated sources for food and drink.

Numerous investigations have linked cholera transmission to drinking water drawn from shallow wells, rivers, or streams, and, in one instance, to commercially bottled spring water. Ice made from contaminated water has also been implicated. In some cities of the developing world, large municipal water systems distribute unfiltered and unchlorinated water through leaky pipes at low pressure. In some areas, people dig down to the water main and puncture it to get water to drink. The hole in the pipe may be plugged with a rag or by another ineffective method. If the pressure drops within the pipe, it can cause back-siphonage and resultant contamination. Similarly, many home water storage containers allow contamination to occur. Introducting safer water storage containers in the home and improving the integrity of municipal water systems have become priorities in many areas now affected by epidemic cholera.

Food is the other important means of cholera transmission. Seafoods have repeatedly been a source of cholera, particularly raw or undercooked shellfish harvested from sewage-contaminated beds or from environments where *V. cholerae* O1 occurs naturally. Crab, shrimp, oysters, clams, and dried fish have all been implicated in foodborne outbreaks. Although *V. cholerae* O1 is easily killed by drying, sunlight, and acidity, it grows well on a variety of moist foods from which other competing organisms have been eliminated by previous cooking. Cooked rice is an excellent growth medium, as are lentils, millet, and other moist foods with neutral pH. In one investigation in Africa, leftover rice served with an alkaline peanut sauce transmitted cholera. The same rice served with acidic tomato sauce was not a vehicle for spread of infection. Fruits and vegetables grown in sewage and eaten without cooking or other decontaminating procedures are probably vehicles of cholera transmission. Freezing food or drink does not prevent cholera transmission; outbreaks in 1991 were traced to ice made from contaminated water, salad made with frozen crab, and frozen coconut milk used as a dessert topping.

Person-to-person spread through direct contact, as by shaking hands or touching or by taking care of a patient, has not been shown to occur. Outbreaks on crowded hospital wards are likely to be due to contaminated food or water. Likewise, outbreaks following the funeral of a cholera patient appear to be caused by eating contaminated foods served at the wake, often prepared by the same persons that prepared the body for burial.

F. Surveillance

Cholera surveillance starts with clinicians and microbiologists who are alert to the possibility of cholera and obtain appropriate cultures for confirmation when suspect illness occurs. Initial suspected cases should be reported immediately to local, state, or regional health departments. Isolates that appear to be *V. cholerae* should immediately be submitted to a reference laboratory for identification and confirmation. All *V. cholerae* isolates should be tested to determine if they are of the O1 serogroup. Isolates of *V. cholerae* O1 associated with sporadically occurring infections and a sample of those identified in an epidemic should be tested for cholera toxin production. Isolates of *V. cholerae* O1 from persons with severe watery diarrhea require prompt notification of the appropriate public health authorities, even before toxin testing is confirmed, so that the source of infection can be investigated and appropriate control measures can begin.

G. Cholera Vaccine

A parenteral cholera vaccine has been available for a number of years. It is a relatively ineffective injectable preparation of killed bacterial cells. This vaccine decreases the risk of clinical cholera in the recipient by only

50%, and the duration of protection is limited to 3 to 6 months. Moreover, the vaccine does not seem to decrease the severity of disease in persons who contract cholera after vaccination. This vaccine does not prevent asymptomatic infection, nor does it prevent infection in persons in close contact with cholera patients. Mass immunization with this cholera vaccine has not proved helpful in the control of epidemic cholera. The vaccine is not recommended for public health use or for travelers to areas with endemic cholera. Proof of vaccination is currently not officially required by any country. Immunization with an ineffective vaccine creates a false sense of security which may interfere with the consideration of more effective prevention measures.

Although the existing cholera vaccine has no practical value, several promising experimental vaccines are being developed. Included in these are two oral formulations: a killed whole-cell vaccine containing the B subunit of cholera toxin, and a live attenuated vaccine. A cholera vaccine that could induce sustained protection against serious illness and, ideally, against asymptomatic infection may have important public health value.

H. Prevention Strategies

Efforts to control epidemics usually occur in several stages. Immediate, short term, emergency control measures include 1) improving the capacity to diagnose, treat and monitor the spread of epidemic cholera; 2) educating the public in simple prevention measures, such as boiling or treating drinking water and avoiding high-risk foods; and 3) epidemiologic investigation to identify specific control measures. Intermediate control measures to be implemented over several months or years include a variety of sustainable and relatively inexpensive interventions, such as introducing safer methods for water storage in the home, using economical and practical methods for local disinfection of water, and providing a safer food supply, including improving the safety of foods and beverages sold by street vendors. Epidemic cholera is likely to persist until long-term sanitary reform measures occur in affected areas. These measures include maintaining and upgrading safe water supplies and distribution systems, constructing effective sewage treatment plants, and providing an effective public health infrastructure to guide the transition to an environment free from the risk of cholera.

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II. The Role of the Public Health Laboratory

A. General Considerations

An understanding of the use of laboratory results is essential in guiding the selection of any laboratory tests. This is particularly important with cholera because in areas where the disease is endemic, cholera may be diagnosed with acceptable accuracy on the basis of clinical symptoms alone. Likewise, cholera may be effectively treated without laboratory confirmation of the etiologic agent. However, early in an outbreak, laboratory confirmation of the etiologic agent is required because other secretory diarrheal illnesses, such as those caused by toxigenic *Escherichia coli* or rotavirus, can mimic cholera. Moreover, because most infections (approximately 75%) with *V. cholerae* O1 are asymptomatic, and cholera gravis (life-threatening dehydration) develops in only about 2% of cholera patients, the diagnosis of cholera may be missed if it is based solely on symptoms, particularly in the early stages of an outbreak or where few cases occur.

During a cholera outbreak, laboratory efforts should be directed toward resolving critical public health issues rather than toward processing a large number of clinical specimens that yield little new information. Laboratory priorities change during the course of a cholera epidemic. In the beginning of an outbreak, confirmation of the etiologic agent is required for suspected cases. Once a cholera epidemic has been established, confirming each clinically diagnosed case is less important, and laboratory efforts should shift to investigating the extent of the epidemic, the source of infection, and the development of antimicrobial resistance. After the epidemic wanes, laboratory confirmation of suspect cases is important for defining the end of the epidemic and guiding public health decisions.

B. When a Threat of Epidemic Cholera is Recognized

In a region threatened by epidemic cholera, the public health laboratory plays a central role in detecting its introduction. Early detection of cholera cases facilitates the selection of appropriate control activities. Laboratory-based surveillance is performed using several types of samples:

1. Clinical samples from "cholera-like illness" at sentinel clinics or hospitals in the absence of a cholera outbreak

Regular sampling of specimens from highly suspect cases can be done periodically, depending on laboratory resources and availability of clinical sites. The definition of suspect cases to be examined for *V. cholerae* should be agreed upon in advance. A patient presenting with severe watery diarrhea and dehydration requiring intravenous therapy should be suspected of having cholera and should be a candidate for surveillance culture. Available resources and the frequency of suspect cases should be

taken into account in performing cultures. Arrangements can be made to collect rectal swabs from all patients with cholera-like illness at several clinical sites, or to sample a subgroup of patients, such as all those with acute diarrhea seen on a particular day of the week or a specific number of patients per month. It is important to plan for an appropriate way of transporting specimens to the laboratory. Examining a limited number of carefully selected specimens is more effective than examining a large number of poorly chosen specimens that may have been improperly handled.

2. Confirmation of cases in the early stages of an outbreak of "cholera-like illness"

Cultures and serologic tests may be done on specimens from household contacts of initial case-patients, and heightened surveillance should be considered for a limited time in the area where an initial case has been confirmed. Such efforts can rapidly determine if the first case was an isolated event or the beginning of an outbreak.

3. Surveillance of sewage collection points

The Moore swab (see Chapter V, "Examination of Environmental Samples") is a simple, reliable, and sensitive method to identify infected individuals in the population served by a sewage collection system. Repeated sampling at central points at 1- to 2-week intervals can efficiently identify infections, symptomatic or asymptomatic, in the area. However, during an epidemic, when swabs are routinely positive, continued sampling offers little additional information and can be discontinued.

4. Laboratory confirmation of *V. cholerae* isolates

If a specimen in the pre-epidemic phase yields *V. cholerae* O1, the isolate should be sent to a reference laboratory for confirmation and characterization. Confirmation of the O1 antigen, the serotype, and the production of cholera toxin is critical for confirming the presence of cholera in the area. A limited number of isolates should be biochemically identified, biotyped, and tested for antimicrobial resistance. Further characterization of isolates, such as molecular subtyping, may help determine the origin of the isolate.

C. During a Cholera Outbreak

Once cholera cases have been confirmed in an area and ongoing transmission is documented either through clinical or environmental surveillance, laboratory efforts aimed at detecting *V. cholerae* O1 infections should be significantly reduced. Not all suspect cases need confirmation, and those that are confirmed do not require extensive characterization. Because the epidemic strain is far more common than nontoxigenic O1 strains, there is little value in testing all isolates for toxin production or

for performing tests other than agglutination with O1 antiserum. Instead, the resources of the public health laboratory should be used for the following:

1. Monitoring development of antimicrobial resistance

Periodic examination of a small number of isolates can detect emerging antimicrobial resistance. Resistance may emerge locally; therefore, small periodic surveys throughout the affected area should be considered.

2. Special epidemiologic investigations

During cholera epidemics, focused field investigations help determine sources of infection and routes of transmission and the rate of spread to family contacts.

Case-control investigations are most precise if both patients and controls can be examined for current or recent infection. Current infection can be documented in patients by culturing specimens from them as they come for treatment. Also, in areas newly affected by an epidemic, serum samples from healthy controls can be analyzed for vibriocidal antibody titers, since potential controls with elevated vibriocidal antibodies indicating recent infection may be excluded from analysis.

Surveys in the households of case-patients can determine if intrafamilial transmission is occurring and interventions, such as household education or household chemoprophylaxis, are warranted.

3. Defining the magnitude of the epidemic and improving the interpretation of surveillance data

Serologic surveys conducted periodically during an epidemic can help determine the number of infections in the population and the proportion that are symptomatic. Results of cultures taken from a sequential series of 50 to 100 patients that meet the case definition used during an epidemic can determine the predictive value of the definition. This will confirm the accuracy of the case definition used for surveillance purposes. If, for example, 80% of patients who meet the case definition have culture-confirmed infection, the predictive value of the definition is high, and patients meeting these criteria may be presumed to have cholera in the absence of culture confirmation.

4. Measuring the impact of control measures

Laboratory surveillance data can determine the effectiveness of control measures. If prevention measures are targeted at specific vehicles of transmission, laboratory tests which document the success or failure of current efforts to disinfect those vehicles can be used as indicators of the efficiency of specific control measures, rather than simply culturing vehicles for *V. cholerae* O1. Evidence of adequate chlorination of water supplies and documented absence of fecal coliforms in water, foods, and

beverages are more reassuring than the failure to detect *V. cholerae* O1 in samples of these items.

D. Defining the Duration of the Epidemic

A substantial decrease in the number of clinically defined cholera cases in a population may represent a temporary seasonal decline, transition to an endemic state, or complete disappearance of cholera from the population. Transmission may cease altogether in some regions, and persist at lower levels in others. Because epidemic cholera often decreases sharply in cooler seasons and returns the following summer, declarations that an area is cholera-free may be premature. At this point, targeted laboratory surveillance can help define the situation. As in the pre-epidemic period, periodic sampling of persons with severe "cholera-like illness" and of central sewage collection points can again be used with great sensitivity to determine the presence or absence of the epidemic strain. Not until 12 months have passed without evidence of *V. cholerae* O1 can an area be declared cholera-free with confidence. A report can state that no cases of cholera have been detected since a specific date without declaring that an area is cholera-free.

E. Special Problems

1. Retrospective diagnosis of a suspected outbreak

If a population is reached after a suspected cholera outbreak has subsided, serum samples may be collected for assay of vibriocidal antibody titers. Sera may be collected from a limited number of "typical" patients and healthy controls. Collecting control sera in the same village permits comparison of vibriocidal titers of the "ill" group and the "healthy" group. The choice of serologic assay depends on the timing of specimen collection (see Chapter VIII, "Detection of Patient Antibodies to *V. cholerae* O1 and Cholera Toxin"). Vibriocidal titers begin to rise several days after exposure, usually peaking by 10 to 21 days, begining to decline within 1 month, and returning to baseline levels after about 1 year. Anti-cholera toxin antibodies peak 21 to 28 days after exposure and remain elevated for more than a year after infection.

2. Environmental sampling

Methods for sampling foods, water, and other environmental specimens for *V. cholerae* O1 are labor-intensive and relatively insensitive and can rapidly deplete laboratory resources without yielding clearly interpretable results. Culture-negative environmental samples may mean that the specimens were collected too late or were mishandled. If several different types of samples yield *V. cholerae* O1, it may not be clear whether the food or water caused the illness or were contaminated by the infected persons.

In general, broad environmental surveys are not recommended. However, if a specific vehicle of transmission has been identified epidemiologically, targeted sampling of that suspected vehicle can yield useful information. Similarly, once control measures have been taken to reduce the contamination of the vehicle, microbiologic assessment can determine the success of the intervention.

F. Summary

In summary, the public health laboratory can provide critical information in defining the beginning of a cholera epidemic, monitoring resistance and other changes in the epidemic strain, and defining the course of the epidemic. Collaborating with epidemiologists, the public health microbiologist can support efforts to determine the sources of infection and measure the effectiveness of control measures. Many critical questions can be answered by careful use of laboratory resources. Clarifying the precise questions to be answered, and giving careful attention to sample selection, specimen transportation, and the efficient use of diagnostic tests can prevent depletion of laboratory resources by tests of questionable epidemiologic value.

Reference

Global Task Force on Cholera Control. Guidelines for cholera control. Geneva: World Health Organization; 1992. publication no. WHO/CDD/SER/80.4 Rev 4.

III. Collection and Transport of Patient Specimens

Inadequately or improperly collected or transported specimens can hamper the efforts of the laboratory to recover *Vibrio cholerae* O1. Therefore, it is crucial to any laboratory investigation to collect suitable specimens, use an appropriate transport medium for fecal specimens, and transport specimens to the laboratory in a timely manner (see Table III-1).

A. Collection of Specimens

1. Fecal specimens

Fecal specimens should be collected in the early stages of any enteric illness, when pathogens are usually present in the stool in highest numbers, and before antibiotic therapy has been started.

Collection of stool

Collect stools from patients in clean (no disinfectant or detergent residue) containers with tight-fitting, leak-proof lids. Specimens should not be collected from bedpans, as they may contain residual disinfectant. A rectal catheter may be used to collect liquid stool from suspected cholera patients. A sterile catheter is lubricated with sterile liquid, inserted past the rectal sphincter, and gently moved into the intestine until liquid stool begins to flow from the end of the catheter.

Unpreserved stool should be refrigerated if possible and processed within a maximum of 2 hours after collection. Any drying of the specimen or a shift to acid pH will significantly reduce the numbers of viable organisms. If the specimen will be kept for longer than 2 hours before processing, a sterile cotton- or polyester-tipped swab should be immersed into the stool and placed in Cary-Blair transport medium (see Section B of this chapter for instructions on inoculation of Cary-Blair). If mucus and shreds of intestinal epithelium are present, these should be sampled with the swab. Specimens preserved in transport medium need not be refrigerated unless they are likely to be exposed to elevated temperatures (>40°C).

Collection of rectal swabs

Moisten a sterile cotton-tipped swab with Cary-Blair transport medium (lubricants must not be used), insert the swab through the rectal sphincter into the anal canal, and rotate several times. Examine the swab for evidence of fecal material. Place the swab into the tube of Cary-Blair medium and transport to the laboratory at room temperature or refrigerated (4°C).

Table III-1. Collection of specimens for laboratory diagnosis of cholera

Collection instructions	Fecal specimens	Serum specimens
When to collect	During period of active diarrhea (as soon after onset of illness as possible)	Acute-phase serum within 3 days after onset if possible; convalescent-phase serum 10-21 days after onset[a]
How much to collect	Rectal swab or swab of fresh stool	10-15 ml whole blood from adults or 3-5 ml from children
Method of collection	Stool or rectal swab placed in Cary-Blair transport medium	Collect blood in tubes without anticoagulants; clot and centrifuge; place serum in sterile tube
Storage of specimen after collection	Store at room temperature or refrigerate at 4°C	Immediately refrigerate or freeze serum (do not freeze whole blood)
Transportation	Seal samples to prevent leakage; ship in durable container by overnight delivery	Place sealed samples on ice packs or dry ice; ship in insulated box by overnight delivery

[a] Vibriocidal antibody titers begin to rise several days after onset and peak by 10-21 days.

2. Serum for antibody studies

Obtain two serum specimens (an acute-phase specimen and a convalescent-phase specimen). Obtain the acute-phase serum specimen as close to the time of onset of illness as possible (ideally within 0 to 3 days after onset of illness) and the convalescent-phase serum specimen 10 to 21 days after the onset of illness. Indicate the date the blood specimen was drawn and the date of onset of disease in addition to the name and age of the patient.

Collect blood specimens from adults (10 to 15 ml) and from children (3 to 5 ml) in tubes that do not contain anticoagulants. Allow blood to clot. Centrifuge the blood and send only the serum for analysis. If no centrifuge is available, store the blood specimens in a refrigerator until a clot has formed; then carefully remove the serum with a Pasteur pipette, avoiding red blood cells. Place the cell-free serum into a sterile screw-capped tube. If the serum is to be held for several days, freezing is preferable to refrigeration to prevent bacterial growth. If possible, ship frozen specimens on dry ice. However, if serum contains red blood cells, it should not be frozen because this will cause lysis of the cells and make analysis difficult. If the serum has been drawn off without centrifuging using the clotting technique described above, ship the specimens refrigerated (not frozen), so that they can be centrifuged to remove any remaining red blood cells before freezing.

B. Transport Media

1. Cary-Blair

Cary-Blair transport medium is the medium of choice for the preservation and transport of *V. cholerae* because its high pH (8.4) is optimal for this species and its semisolid consistency provides for ease of transport. Cary-Blair medium is stable and can be stored after preparation for up to 1 year in tightly sealed containers. It can be used to transport many enteric pathogens besides *V. cholerae*. (See Chapter XI, "Preparation of Media and Reagents" for instructions on how to prepare Cary-Blair medium.)

Inoculation of Cary-Blair transport medium

After collecting the specimen, aseptically insert one or two swabs into the tubed medium (Figure III-1). Keep the head of the swab submerged in the medium. Aseptically break off the end portion of the swab stick that has been touched by the fingers. Replace the screw cap tightly. Transport or store inoculated medium at room temperature (25°C) or refrigerated (4°C).

2. Other transport media

Alkaline peptone water (APW) may be used to transport *V. cholerae* for short periods if Cary-Blair is not available. APW should not be used if

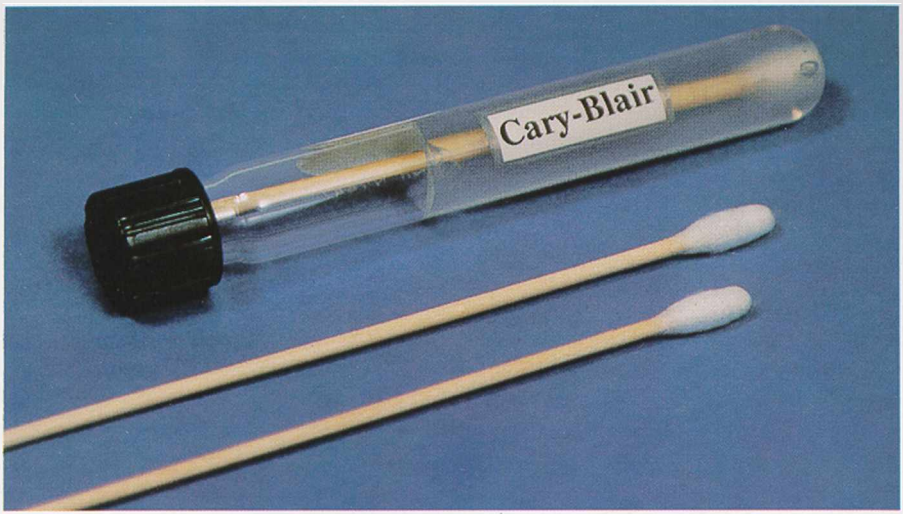

Figure III-1. Cary-Blair semisolid transport medium

subculture will be delayed more than 6 hours from the time of collection because other organisms will overgrow vibrios after 6 hours.

Amies' (pH 7.4) or Stuart's (pH 7.3) transport media may be used to transport specimens if Cary-Blair is not available but only for short periods (1-2 days) because the pH of these media is not optimal for *V. cholerae*. Buffered glycerol saline is not suitable for transport of *V. cholerae* and must not be used.

C. Unpreserved Specimens

If transport media cannot be used, one option is to soak a piece of filter paper, gauze, or cotton in liquid stool and place it into a plastic bag. The bag must be tightly sealed so that the specimen will remain moist and not dry out. Adding several drops of sterile saline to the bag may help prevent drying of the specimen. Refrigeration during transport is desirable but not necessary.

D. Transport of Specimens

The transport or shipment of diagnostic specimens by public or commercial delivery systems may be subject to local or national regulations. Transport of specimens should not present a hazard to humans or the environment and should protect the viability of suspected pathogens. For instructions about the international shipment of diagnostic specimens and etiologic agents, see Chapter XII, "Storage and Shipment of Isolates."

The following guidelines are suggested for the transport of diagnostic specimens in the absence of specific regulations:

- Place each specimen in an appropriate primary container (test tube, stool cup, transport medium, serum vial).
- Place primary containers in a durable covered waterproof secondary container with sufficient blotting material (e.g., cotton wool, paper towels) to absorb any leakage from the primary container.
- Specimens may be refrigerated by placing them in an insulated box with frozen refrigerant packs (these may be commercial or homemade). If specimens are refrigerated with wet ice instead of refrigerant packs, water from the melting ice should not seep into the specimen tubes or leak from the secondary container. If wet ice is used, place the specimen containers in waterproof plastic bags that can be tightly sealed.
- Frozen specimens can be kept frozen only by shipping them on dry ice (solid CO_2). Use enough dry ice to keep the specimen frozen until it is received at the laboratory that will process it (usually about one-third to one-half of the shipping container). Glass tubes should not come into direct contact with dry ice; cushion specimens with a layer of packing paper or other buffering material between the tubes and the dry ice.
- Pack specimens on dry or wet ice securely, so that they will not roll loosely in the container after the ice has dissipated.
- Specimens that are not refrigerated or frozen should still be protected against possible extremes of heat or cold.
- Select the most rapid and reliable means of transportation to avoid delays in shipment. If air transport is used for frozen specimens, obtain prior approval from the airline authorizing the use of dry ice, because CO_2 gas is released as it melts. Schedule delivery during business hours on a weekday if possible.
- If specimens are shipped by mail, pack samples according to national and/or international postal requirements (see Chapter XII.) Be sure to attach all necessary information and documentation for each specimen.

References

1. World Health Organization. Manual for the Laboratory Investigations of Acute Enteric Infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.
2. CDC. Recommendations for the collection of laboratory specimens associated with outbreaks of gastroenteritis. MMWR 1990;39 (No. RR-14).
3. Cary SG, Blair EB. New transport medium for shipment of clinical specimens. I. Fecal specimens. J Bacteriol 1964; 88:96-8.
4. Global Task Force on Cholera Control. Guidelines for cholera control. Geneva: World Health Organization, 1992; publication no. WHO/CDD/SER/80.4 rev 4.

IV. Isolation of *Vibrio cholerae* from Fecal Specimens

Although *V. cholerae* O1 will grow on a variety of commonly used agar media, isolation from fecal specimens is more easily accomplished with specialized media. Alkaline peptone water (APW) is recommended as an enrichment broth, and thiosulfate citrate bile salts sucrose (TCBS) agar is the selective agar medium of choice for isolating *V. cholerae* O1. In certain instances (for example, when the patient is in very early stages of illness and is passing liquid stool), it may not be necessary to enrich specimens or use selective plating media. However, enrichment broth and a selective plating medium should always be used with convalescent patients, suspected asymptomatic infections, environmental specimens, and whenever high numbers of competing organisms are likely to be present in the specimen.

A. Enrichment in Alkaline Peptone Water

Vibrio spp. grow very rapidly in APW, and at 6 to 8 hours will be present in greater numbers than non-*Vibrio* organisms. Enrichment in APW enhances the isolation of *V. cholerae* O1 when few organisms are present, as in specimens from convalescent patients and asymptomatic carriers.

A number of other broth media have been described for enrichment of *V. cholerae*. These include Monsur's enrichment medium which contains Trypticase, potassium tellurite, and sodium taurocholate (bile salts). A modification of APW, in which potassium tellurite is added in concentrations of 1:100,000 to 1:200,000, is sometimes used. An enrichment medium containing a selective agent may not offer any advantage over APW if it is used with a short incubation time (6 to 8 hours).

B. Selective Plating Media

1. Thiosulfate citrate bile salts sucrose agar

TCBS is the medium of choice for the isolation of *V. cholerae* and is widely used worldwide. TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective (Table IV-1). However, it has a relatively short shelf life once prepared (3 to 5 days) unless plates are carefully protected against drying. TCBS is subject to lot-to-lot and brand-to-brand variations in selectivity, and growth on this medium is not suitable for direct testing with *V. cholerae* O1 antisera.

TCBS agar is green when prepared. Overnight growth (18 to 24 hours) of *V. cholerae* will produce large (2 to 4 mm in diameter), slightly flattened, yellow colonies with opaque centers and translucent peripheries (Figure IV-1). The yellow color is caused by the fermentation of sucrose in the medium. Sucrose nonfermenting organisms, such as *V. parahaemolyticus,* produce green to blue-green colonies. Suspicious colonies

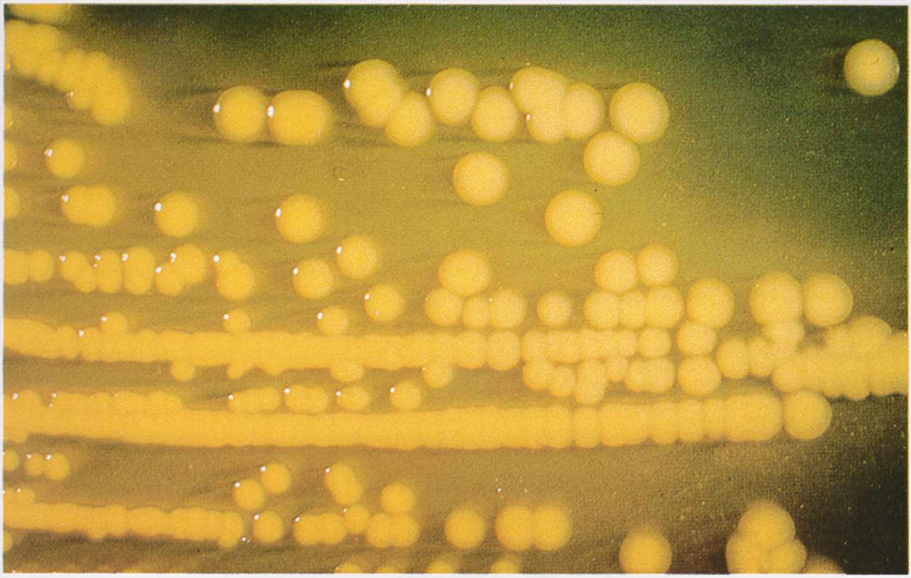

Figure IV-1. Overnight colonies of *V. cholerae* on TCBS agar are large (2-4 mm) and yellow because of the fermentation of sucrose. They are characteristically round, smooth, glistening, and slightly flattened.

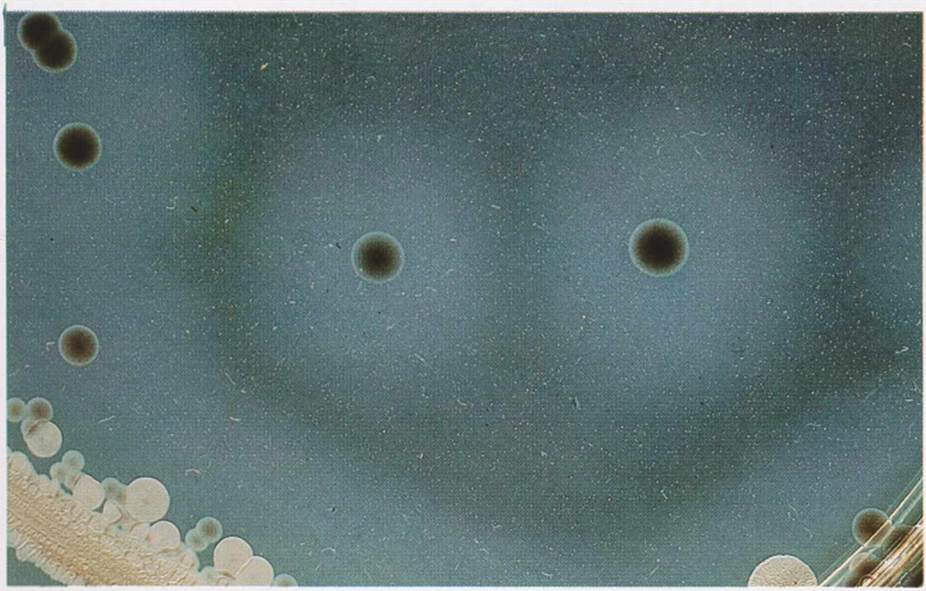

Figure IV-2. On TTGA medium, colonies of *V. cholerae* are grey, flattened, and are surrounded by a cloudy halo formed by the production of gelatinase.

for further testing should be subcultured to a noninhibitory medium, such as gelatin agar, heart infusion agar (HIA), Kligler's iron agar (KIA), or triple sugar iron agar (TSI).

2. Taurocholate tellurite gelatin agar (TTGA or Monsur's agar)

TTGA is a selective and differential agar specifically designed for the isolation of *V. cholerae*. TTGA has a relatively long shelf life after preparation, and growth directly from the medium may be used for oxidase and agglutination tests (Table IV-1). The disadvantages of this medium are that it is not commercially available, and overnight colonies of *V. cholerae* on TTGA tend to be smaller (1 to 2 mm) than those from TCBS agar. Potassium tellurite, which is added to the medium to increase selectivity, also varies in its quality, and each lot should be titrated to determine the optimal concentration to use in TTGA medium (see Chapter XI, "Preparation of Media and Reagents").

Overnight growth of *V. cholerae* on TTGA agar appears as small, opaque colonies with slightly dark centers (Figure IV-2). After 24 hours, the centers of the colonies become darker, and eventually the entire colony becomes "gunmetal" grey in color. In addition to the dark coloration, which is due to the reduction of tellurite, there is also a opaque zone around colonies which resembles a halo. The halo effect, which is due to the production of the enzyme gelatinase, can be intensified by brief (15- to 30-minute) refrigeration of the plate. Because many members of the genus *Vibrio* have similar characteristics on TTGA, additional tests (antisera and/or biochemicals) are necessary to screen isolates from this medium.

Table IV-1. Selective plating media for *V. cholerae*

Medium	Colony morphology	Colony size	Commercially available	Auto-claved	Direct testing of growth off of plate[a]
TCBS	Yellow, shiny	2-3 mm	Yes	No	No
TTGA	Grey, flattened opaque zone around colony	1-2 mm	No	Yes	Yes
Mac-Conkey[b]	Colorless to light pink	1-3 mm	Yes	Yes	No

Note: TCBS = thiosulfate citrate bile salts sucrose agar; TTGA = taurocholate tellurite gelatin agar.

[a] Direct testing for agglutination in antisera or oxidase reaction.

[b] Not all strains of *V. cholerae* O1 will grow on MacConkey agar.

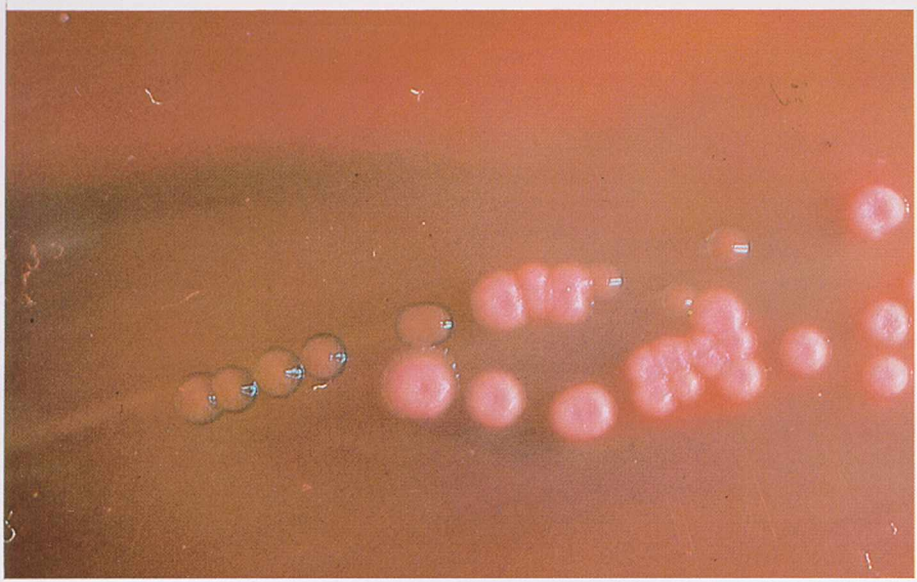

Figure IV-3. Overnight growth of *V. cholerae* on MacConkey agar appears as small (1- to 3-mm), translucent, colorless-to-light pink (lactose-negative) colonies.

3. MacConkey (MAC) agar

MAC is used widely to isolate members of the *Enterobacteriaceae* and will also support the growth of some but not all strains of *V. cholerae*. Overnight colonies of *V. cholerae* on MAC tend to be small to moderately sized (1 to 3 mm) and usually appear as lactose-negative or slightly pink, often resembling colonies of "late" or "slow" lactose-fermenting organisms (Table IV-1; Figure IV-3). Suspicious colonies should be subcultured to noninhibitory media for further testing.

C. Nonselective Plating Media

1. Gelatin agar (GA)

GA is a good nonselective growth medium for *V. cholerae*. Gelatinase production, a characteristic of vibrios in general, may be determined on GA and is indicated by the production of an opaque zone around colonies which resembles a halo. The halo effect can be intensified by brief (15- to 30-minute) refrigeration of the plate. Colonies of *V. cholerae* on GA are smooth, opaque, white, and 2 to 4 mm in diameter after overnight incubation at 35° to 37°C. When viewed with obliquely transmitted light with 10x to 20x magnification, colonies may appear finely granular and iridescent with a greenish-bronze sheen. Colonies from this medium may be tested directly for agglutination with antisera as well as oxidase and string test reagents. Salt-free GA may be used as a screening medium to rule out halophilic (salt-requiring) marine vibrios resembling *V. cholerae,*

which are frequently isolated from seafood and environmental specimens. See Chapter XI, "Preparation of Media and Reagents," for special instructions for preparation of gelatin agar.

2. Meat extract agar (MEA, or alkaline nutrient agar)

MEA is similar to GA in its ability to support the growth of *V. cholerae*. However, unlike GA, MEA does not produce colonies with differential characteristics. Colonies of *V. cholerae* on MEA are 2 to 4 mm in diameter after overnight incubation and are smooth, opaque, and cream colored. When viewed with oblique light with 10x to 20x magnification, colonies may appear finely granular and iridescent with a greenish-bronze sheen. The oxidase test, string test, and screening with antisera may be performed directly with suspicious growth on MEA plates.

D. Isolation and Presumptive Identification

1. Direct inoculation of selective plating media from fecal specimens

Inoculate highly selective media (TCBS, TTGA) with a heavy inoculum from liquid stool, fecal suspension, or a rectal swab (Figure IV-4). With media of low selectivity (GA, MEA, blood agar), use a light inoculum. The inoculum should be streaked with a wire loop to give a large number of isolated colonies. It is not necessary to flame the loop between streaking different quadrants on the plate. Media of high selectivity require more cross streaking into previous quadrants than media with lower selectivity. After inoculation, incubate plates for 18 to 24 hours at 35° to 37°C.

2. Inoculation of APW from fecal specimens

APW can be inoculated with liquid stool, fecal suspension, or a rectal swab (Figure IV-4). The stool inoculum should not exceed 10% of the volume of the broth. Incubate the tube with the cap loosened at 35° to 37°C for 6 to 8 hours. After 6 to 8 hours' incubation, subcultures to TCBS should be made with one to two loopfuls of APW from the surface and topmost portion of the broth, since vibrios preferentially grow in this area. Do not shake or mix the tube before subculturing. If the broth cannot be subcultured after 6 to 8 hours of incubation, subculture at 18 hours to a fresh tube of APW. This second tube should be subcultured to a solid medium after 6 to 8 hours of incubation.

3. Isolation of suspicious colonies from plating media

Select several suspect colonies from the TCBS plate and use these to inoculate HIA slants or another nonselective medium. Do not use nutrient agar because it has no added salt and does not allow optimal growth of *V. cholerae*. Incubate at 35° to 37°C.

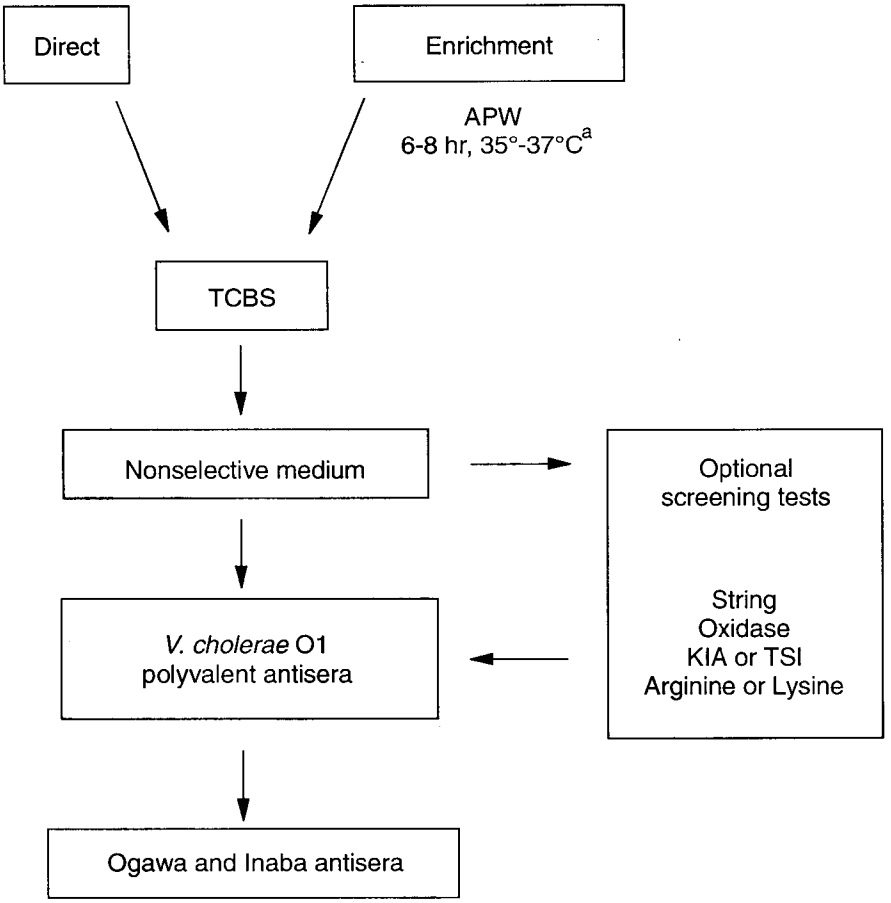

Figure IV-4. Procedure for recovery of *Vibrio cholerae* O1 from fecal specimens

[a] If the APW cannot be streaked after 6-8 hours of incubation, subculture at 18 hours to a fresh tube of APW; incubate for 6-8 hours, and then streak to TCBS.

4. Slide agglutination

Fresh growth of suspect *V. cholerae* on a nonselective agar medium may be tested in *V. cholerae* O1 polyvalent antiserum. Usually after 5 to 6 hours of incubation, growth on the surface of the slant is sufficient to perform slide serology with *V. cholerae* O1 polyvalent antisera; if not, reincubate overnight. Isolates that agglutinate in polyvalent antiserum to the O1 serogroup are presumptively identified as *V. cholerae* O1 (see Chapter VI, "Laboratory Identification of *V. cholerae*," for description of the slide agglutination method). Presumptive *V. cholerae* O1 may be confirmed with agglutination in either monovalent Ogawa or Inaba antisera, but confirmation may not be necessary for all isolates, particularly when the supply of antisera is limited. The minimum identification of *V. cholerae* O1 requires only serologic confirmation of the presence of O1 serotype antigens with suspect isolates. However, a more complete characterization of the organism is sometimes necessary and may include toxin or hemolysis testing, as well as determination of antimicrobial sensitivity, biochemical identification, biotype, or molecular subtype. These types of tests should be performed only on selected isolates, such as those recovered early in an outbreak or during surveillance in areas threatened by epidemic cholera. Only isolates that are serologically confirmed to be *V. cholerae* O1 should be further characterized. (See Chapter II for a discussion of when further characterization of isolates is necessary, and Chapters VI, VII, and IX for a description of these tests.)

5. Biochemical screening tests

Generally for suspect *V. cholerae* isolates from fecal specimens, screening with biochemical tests is not necessary since slide serology with polyvalent O1 antisera is sufficient for a presumptive identification. However, if the supply of antisera is limited, the string and oxidase tests or various other biochemical tests may be useful for additional screening of isolates before testing with antisera. No single screening procedure for *V. cholerae* O1 is ideal for all laboratories and every sample. The laboratorian should select a screening procedure on the basis of available resources (for example, the availability of antisera), the types and numbers of competing organisms likely to be present in the specimens being cultured, and the ability of the selective plating medium to inhibit the growth of those competing organisms.

The string test, using fresh growth from nonselective agar, is useful for ruling out non-*Vibrio* spp., particularly *Aeromonas* spp. (Table IV-2). Oxidase can also be used to screen out non-*Vibrio* spp. such as *Enterobacteriaceae*. Kligler's iron agar (KIA) or triple sugar iron (TSI) agar rules out *Pseudomonas* and certain *Enterobacteriaceae* spp. Arginine is generally more helpful than lysine for screening out *Aeromonas* and certain *Vibrio* spp., but either medium may be used. There is no need to use two biochemicals that rule out the same organism. For example, if arginine is used, it is usually not advantageous to also screen with lysine since they

Table IV-2. Differential characteristics of selected members of the *Vibrionaceae* and *Enterobacteriaceae*

Test	Organism						
	Vibrio cholerae	*Vibrio mimicus*	Halophilic vibrios	*Aeromonas hydrophila*	*Aeromonas veronii*	*Plesiomonas shigelloides*	*Entero-bacteriaceae*
KIA	K/A	K/A	V	V	K/AG	K/A	V
TSI	A/A	K/A	V	V	A/AG	K/A	V
String	+	+	+[a]	-	-	-	-
Oxidase	+	+	+	+	+	+	-
Gas from glucose	-	-	-[b]	+	+	-	V
Sucrose	+	-	V	V	+	-	V
Lysine	+	+	V	V	+	+	V
Arginine	-	-	V	+	-	+	V
Ornithine	+	+	V	-	+	+	V
VP	V	-	V	V	+	-	V
Growth in 0% NaCl[c]	+	+	-	+	+	+	+
Growth in 1% NaCl[c]	+	+	+	+	+	+	+

Note: V = variable reaction.

[a] *V. parahaemolyticus*, *V. cincinnatiensis*, and *V. damsela* give variable reactions.

[b] *V. furnissii* and *V. damsela* are variable for gas from glucose.

[c] Nutrient broth base (Difco Laboratories, Detroit, MI)

generally rule out the same species. Caps on all tubes of biochemicals should be loosened before incubation. This is particularly important for KIA or TSI slants since, if the caps are too tight and anaerobic conditions exist, the characteristic reactions of *V. cholerae* may not be exhibited. (See Chapter VI, "Laboratory Identification of *V. cholerae,*" for description of these biochemical tests.)

E. Rapid Diagnostic Methods

Prompt laboratory diagnosis of cholera is often advantageous for monitoring the spread of the disease and rapidly instituting control measures. Several rapid methods have been developed and used to detect *V. cholerae* O1 directly from stools of acutely ill patients or from enrichment broth. In certain situations, these rapid methods may be practical, but they provide only a preliminary diagnosis. Despite their advantages of speed and (sometimes) simpler requirements for reagents and equipment, for the most part, rapid diagnostic methods cannot entirely replace traditional culture methods. Traditional techniques must be relied on when an isolate is required for further tests, such as assays for cholera toxin production, antimicrobial sensitivity, hemolysis, biotype, or molecular subtyping. Rapid methods may be most useful in field situations where bedside diagnosis is required to monitor the course of an outbreak.

1. Dark-field and phase-contrast methods

Dark-field and phase-contrast microscopy have been used for screening fecal specimens for the presence of *V. cholerae*. With these techniques, liquid stools are microscopically examined for the presence of organisms with typical darting ("shooting star") motility. The observation of characteristic motility can only be considered a screening test, and the diagnostic accuracy is not high when compared with standard culture techniques.

The motility detection method is improved with the use of specific *V. cholerae* O1 antisera. In this method, stools or enrichment broths are examined with and without antisera added. If the addition of polyvalent antisera against *V. cholerae* O1 results in the cessation of motility, as observed by either dark-field or phase-contrast microscopy, the test is considered positive.

The diluents for the antisera and stool must be selected carefully to avoid nonspecific inhibition of motility (for example certain preservatives such as sodium azide or merthiolate used in antisera). Distilled water is not a suitable diluent for stool specimens because it can extinguish the motility of *V. cholerae*. Other disadvantages include the requirements for a dark-field or phase-contrast microscope and a technician experienced in this technique. Despite these disadvantages, this technique has been widely used.

Dark-field procedure

Freshly collected liquid ("rice-water") stool is the specimen of choice for the dark-field procedure. If stool is liquid but not rice-water in nature, it should be diluted 1:1 with saline before direct testing. If freshly collected stool is negative, perform a 6- to 8-hour APW enrichment and retest the enrichment broth. Antisera may be saved by first determining that the stool or enriched specimen is motile before preparing the antiserum-containing specimen for examination. A known motile strain of *V. cholerae* O1 should be used as a positive control.

The following materials are needed:

- Microscope with dark-field condenser (if an oil immersion lens is used [100x], the objective must have an iris diaphragm)
- Polyvalent *V. cholerae* O1 antiserum
- Clean microscope slides (standard size) and cover slips
- Sterile physiological saline or phosphate-buffered saline, pH 7.0 to 8.0

To perform the test, place a drop of polyvalent *V. cholerae* O1 antiserum near the end of a clean microscope slide. Next, mix a drop of freshly collected rice-water stool or a drop of a 6- to 8-hour APW enrichment broth culture with the antiserum, and place another drop of stool or APW at the opposite end of the slide. Place cover slips over the drops at each end of the slide. Using dark-field, examine the drop containing the stool or enrichment broth for "shooting star" motility by using the 40x objective. If this type of motility is detected, examine the antiserum-containing mixture. If the motile organism is *V. cholerae* O1, the motility will be completely extinguished. If the organisms under both cover slips are nonmotile or if there is no difference between the motility of either mixture, the organism is not *V. cholerae* O1.

2. Immunofluorescence

Immunofluorescence techniques using antisera conjugated to fluorescein isothiocyanate have been used to visualize *V. cholerae* O1 cells in a variety of specimen types. Despite the utility of immunofluorescence methods, they are not widely used as primary diagnostic tools because of the requirements for expensive equipment, high quality immunologic reagents, and trained technicians.

3. Latex agglutination

A commercially manufactured slide agglutination kit (Denka Seiken, Tokyo, Japan), developed for serotyping *V. cholerae* O1 isolates, has been used to detect the organism directly in fecal specimens. The kit uses latex particles coated with monoclonal antibodies directed against the A, B, and C antigens of *V. cholerae* O1. During an investigation of an epidemic

of cholera, the kit was evaluated for its ability to confirm the diagnosis of cholera at bedside using rectal swabs. The latex agglutination test detected 63% of culture-positive patients from rectal swabs, but gave false positive results for 12% of culture-negative patients. The sensitivity and specificity of this test with liquid stool specimens have not been determined.

4. Coagglutination

In the coagglutination method, antibodies against *V. cholerae* O1 are bound to the surface of *Staphylococcus aureus* (Cowan 1) cells while retaining their binding capacity and specificity. In a positive reaction, staphylococcal cells are bound together in a lattice-like arrangement caused by the formation of linkages between them created by the binding of the antibody on their surface to the *V. cholerae* bacterial cells.

Problems using this technique have been attributed to substances in stool which nonspecifically inhibit agglutination and lattice formation of staphylococcal cells. Recently, a commercially prepared monoclonal antibody-based coagglutination test (CholeraScreen, New Horizons Diagnostics, Columbia, MD) has been marketed and appears to have overcome these obstacles. Reports of evaluations of the product with culture collections in the United States and with clinical specimens in Guatemala and Bangladesh have been encouraging.

5. Other rapid isolation/identification techniques

Other techniques for the rapid diagnosis of *V. cholerae* O1 include methods based on the multiplication of bacteriophage, addition of antisera to growth medium to precipitate *V. cholerae* cells in broth, and the use of antibody-coated magnetic beads to physically aggregate *V. cholerae* bacterial cells.

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V. Examination of Food and Environmental Samples

Traditionally, water has been considered to be the most important vehicle for cholera transmission. By 1860, after investigations by John Snow and others, it was apparent that sewage-contaminated water sources, such as municipal water supplies, rivers, streams, or wells, were the principal route of disease transmission. Contact with contaminated food can also spread cholera, although most implicated foods are seafoods. In an epidemic setting, water and food are usually contaminated by *Vibrio cholerae* O1 strains from human feces. Thus, for many years, it was believed that the only reservoir of *V. cholerae* O1 was the human intestine and that survival of the organism in the environment was limited. However, during the past 20 years, investigations in Australia and the United States have yielded evidence that both nontoxigenic and toxin-producing strains of *V. cholerae* O1 may be naturally occurring members of the aquatic ecosystem. These data support the concept that toxigenic and nontoxigenic *V. cholerae* O1 strains may have an environmental reservoir, which would have important implications for efforts to control and eradicate cholera.

Like other members of the *Vibrionaceae*, *V. cholerae* O1 can survive in aquatic environments for extended periods and is considered by many to be an indigenous species in estuarine and brackish waters. Various biological and physiochemical factors, such as nutrient content, salinity, temperature, and pH, may influence the growth, survival, and distribution of *V. cholerae* in aquatic environments. The survival time of *V. cholerae* in water may extend from hours to months. The ability of *V. cholerae* to produce chitinase may also contribute to its survival in estuarine environments where plankton and other chitin-containing marine life are plentiful. *V. cholerae* O1 was able to bind to diverse plankton species collected from Bangladesh, where cholera is endemic. Other aquatic biota, such as water hyacinths from Bangladesh waters, have also been shown to be colonized by *V. cholerae* and to promote its growth. This relationship with plankton may be an important aspect of the ecology of *V. cholerae* O1 in cholera-endemic regions of Bangladesh, and this is supported by the fact that seasonal plankton blooms accompany cholera epidemics in Bangladesh. However, although natural bodies of water probably serve as both environmental reservoirs and a means of transmission to humans, attempts to isolate *V. cholerae* from lakes, rivers, streams, and ponds in areas with epidemic disease have not always been successful.

A. Transport of Specimens

Because *V. cholerae* survives better in specimens held at 4°C than in frozen samples, specimens should be held refrigerated by placing them in an insulated box with frozen refrigerant packs (these may be commercial or homemade). If specimens are refrigerated with wet ice instead of refrigerant packs, water from the melting ice should not seep into the speci-

mens or leak from the container. This can be avoided by placing the specimen containers in waterproof plastic bags that can be tightly sealed. Submersion of samples in ice should also be avoided to prevent partial freezing of the samples.

B. Selection of Isolation Methods for Environmental Samples

The selection of the isolation method depends on the type of sample to be cultured. Samples from marine and estuarine environments may contain numerous other *Vibrio* species that grow as well as *V. cholerae* in alkaline peptone water (APW) and on thiosulfate citrate bile salts sucrose agar (TCBS). These samples should be diluted in 10-fold increments to 10^{-3} to reduce the numbers of competing microorganisms. Incubation of APW at an elevated temperature (42°C) inhibits the growth of some competitors, particularly other vibrios that do not grow as well at that temperature. The isolation of *V. cholerae* O1 from estuarine or marine samples may therefore be enhanced because the primary competitors are inhibited at this temperature. If laboratory resources permit, a duplicate set of dilutions may be prepared to be incubated at 42°C. In contrast, specimens such as fresh water or sewage effluent, which contain relatively fewer vibrios and vibrio-like organisms, do not usually require dilution before culturing or incubation at 42°C.

C. Isolation of *V. cholerae* from Sewage

Surveillance using the Moore swab method is a practical and effective technique for detecting *V. cholerae* in sewage (Figure V-1). The swabs can be easily assembled, and when suspended in the intake lines of a municipal sewage system, they can detect *V. cholerae* infections in areas where surveillance of diarrheal illness has failed to detect cholera. The sensitivity of the technique appears not to be dependent on the distance of the source of infection from the sampling site. Using this method to sample all major intake lines of a community sewage system is a simple way to determine whether *V. cholerae* O1 infections are occurring in an area. The assembly, placement, and subsequent handling of Moore swabs for *V. cholerae* sewage surveillance are described below.

Materials (for 10 specimens)

- 10 Moore swabs
- 100 yards nylon fishing line (25 lb test or higher)
- 1 insulated cooler and frozen refrigerant packs for transport
- 10 securely closing suitable containers (500-1000 ml)
- 10 pairs latex gloves
- 3-5 liters of alkaline peptone water (see Chapter XI for formula)

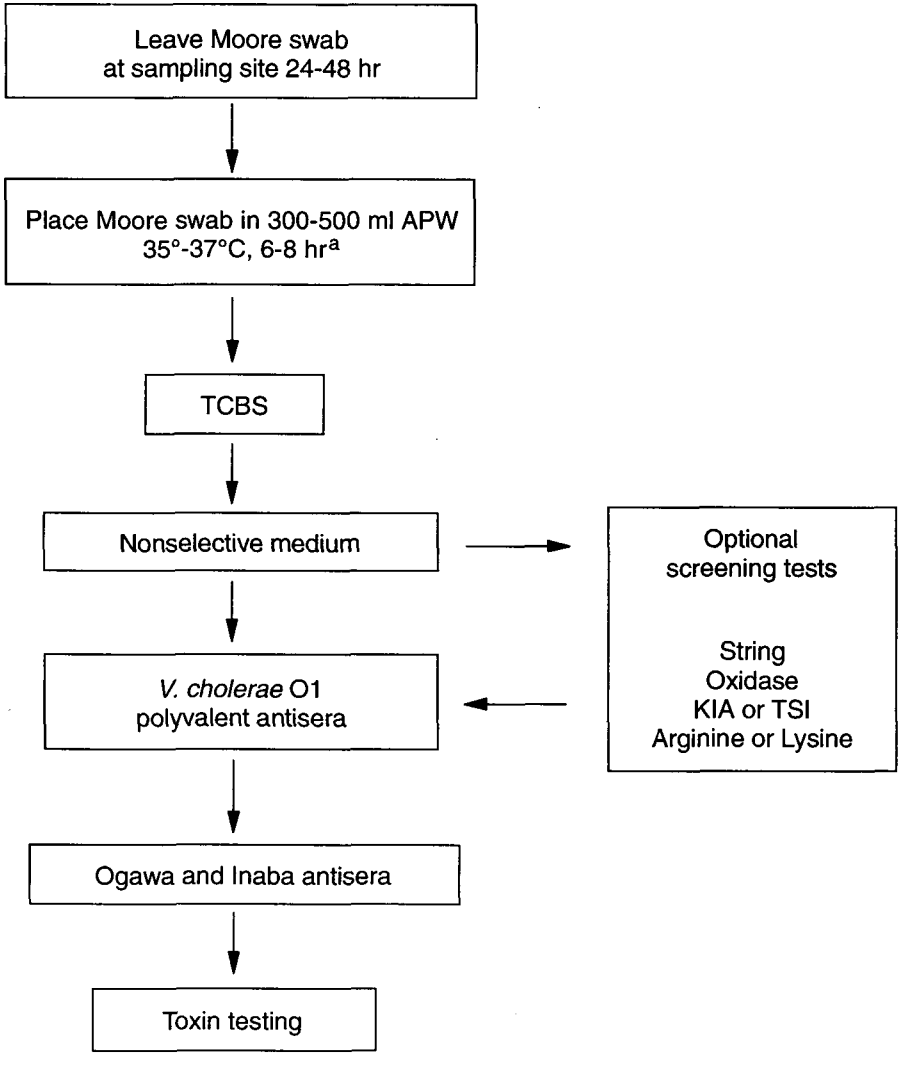

Figure V-1. Moore swab technique for recovering *Vibrio cholerae* O1 from sewage or water

[a] If the APW cannot be streaked after 6-8 hours of incubation, subculture at 18 hours to a fresh tube of APW; incubate for 6-8 hours, and then streak to TCBS.

Assembly of Moore swabs

Moore swabs can be made by cutting pieces of cotton gauze 4 feet long by 6 inches wide (120 cm by 15 cm), folding or rolling the gauze lengthwise several times, and firmly tying the center with fishing line. Sterilization by wrapping in heavy paper and autoclaving before use is optional (Figure V-2).

Placement of Moore swabs

For effective surveillance, place Moore swabs in all main intake lines at the sewage plant or other central locations in the sewage system. The site for swab placement must be carefully evaluated for conditions that could inhibit the recovery of the *V. cholerae* organism. Placement must be upstream of any septic waste dump sites or partially treated recycled sewage to avoid possible contamination with toxic waste. Suspend the swab in the sewage to be tested. In the case of intake lines accessible only by manhole, a piece of wire should be attached to the end of the line to prevent cutting the line when the manhole cover is replaced. The swab should be left in place for 24 to 48 hours.

Collection of specimens

Wear latex gloves and change between specimens to prevent cross-contamination. When Moore swabs, including their attachment line, are removed from the sampling site, they should be placed in securely-closing containers of a suitable size. Label the containers with collection site and

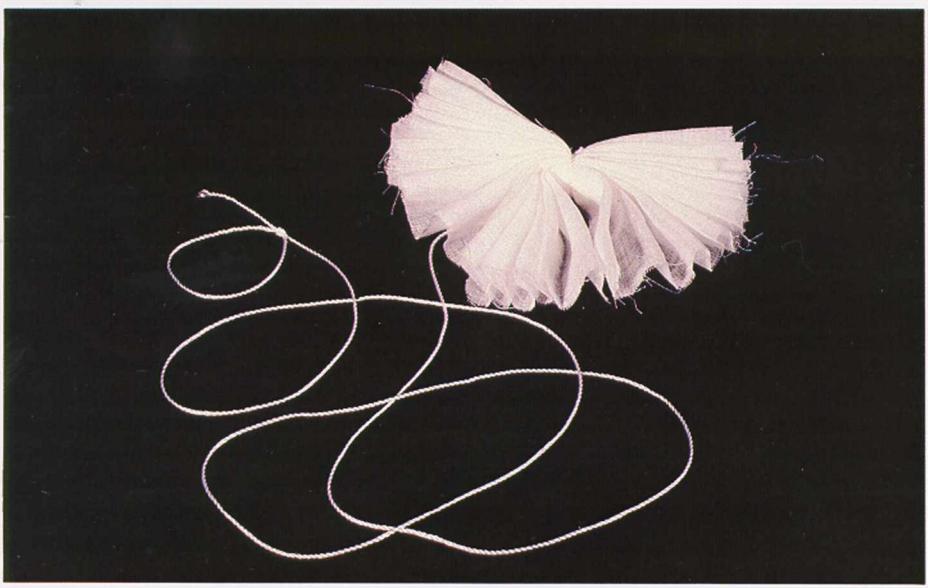

Figure V-2. The Moore swab is a simple device for sampling sewage and contaminated water for the presence of *V. cholerae*.

date. Samples should be transported as quickly as possible to the laboratory in an ice chest with frozen refrigerant packs to prevent possible overheating (see Section A of this chapter for instructions for transport of specimens). If it will be longer than 3 hours between collection of swabs and arrival at the laboratory, the swabs should be placed in APW at the collection site before transport to ensure optimal recovery of *V. cholerae*. APW (300 to 500 ml) should be added to the specimens immediately upon arrival at the laboratory if it was not added at the site. The Moore swab and the associated sample water should make up approximately 10% to 20% of the total volume of the sample with APW added to obtain the optimal ratio of sample to enrichment broth for recovery. Incubate specimens at 35° to 37°C for 6 to 8 hours before plating, as described in Section F of this chapter.

D. Isolation of *V. cholerae* from Water Specimens

All water specimens should be collected in sterile containers and transported to the laboratory under refrigeration (see Section A of this chapter) to prevent overheating. Generally the larger the water sample, the greater the chance of isolating *V. cholerae*. Collecting and processing multiple samples is another way to enhance the chances of isolation. Selection of the isolation method should depend on the type of water sample to be cultured, and salinity of the water source should be the determining factor. For example, ship ballast water, a documented source of *V. cholerae*, should be cultured by the same method as seawater.

1. Direct culture technique

Add 450 ml of water to 50 ml of 10x concentrated APW. An alternate method is to make a 10^{-1} dilution of the water sample in 1x APW (for example, 10 ml of water in 90 ml of APW). The latter method is particularly useful for heavily contaminated samples. If estuarine or sea water samples are cultured, two additional tenfold dilutions should be made in APW to give 10^{-2} and 10^{-3} dilutions. Additional dilutions to 10^{-6} may be done if enumeration is desired (see the MPN technique below). Incubate all dilutions at 35° to 37°C, and plate at 6 to 8 hours as described in Section F of this chapter. If laboratory resources permit, a duplicate set of dilutions may be prepared to be incubated at 42°C. When culturing fresh water samples, further dilutions and incubation at 42°C may not be necessary to enhance isolation since the numbers of competing organisms (particularly *Vibrio* spp.) may be considerably fewer than in marine or estuarine waters.

2. Membrane filter technique

The membrane filter technique is most appropriate for clear water that does not contain debris, mud, or silt. If it is used for cloudy water, a clarifying agent, filter aid, or prefilter to remove suspended materials may be necessary. Filter 100 to 300 ml of sample water (or a larger

amount if possible) through a 0.22- to 0.45-μm membrane (Millipore) filter. Place the filter in 100 ml of APW in a flask. Incubate at 35° to 37°C for 6 to 8 hours and plate to TCBS. If laboratory resources permit, a duplicate specimen may be prepared to be incubated at 42°C. Alternatively, the filter may be placed directly on the surface of a nonselective agar plate (e.g., T_1N_1, heart infusion agar), incubated for 3 hours at 35° to 37°C, then transferred to a TCBS agar plate and incubated for 18 to 24 hours at 35° to 37°C. If estuarine or marine samples are to be cultured, smaller water samples should be filtered, or filters should be placed in larger volumes of APW to dilute the samples adequately.

3. Moore swab

The Moore swab can be used for sampling water as well as sewage, but it is useful only for rivers and flowing water sources and offers no particular advantage over other sampling methods for stationary water sources. As with sewage, the Moore swab should be left at the sampling site for 24 to 48 hours. (Refer to Section C of this chapter for assembly, collection, and examination of Moore swabs.)

4. Spira swab

The Spira swab procedure is a sampling method in which water is filtered through cotton gauze. The gauze is placed in a large plastic bottle (for example, a 500-g media bottle or other equivalent container) with a 2-cm hole cut in the bottom (Figure V-3). The size of the hole in the bottom

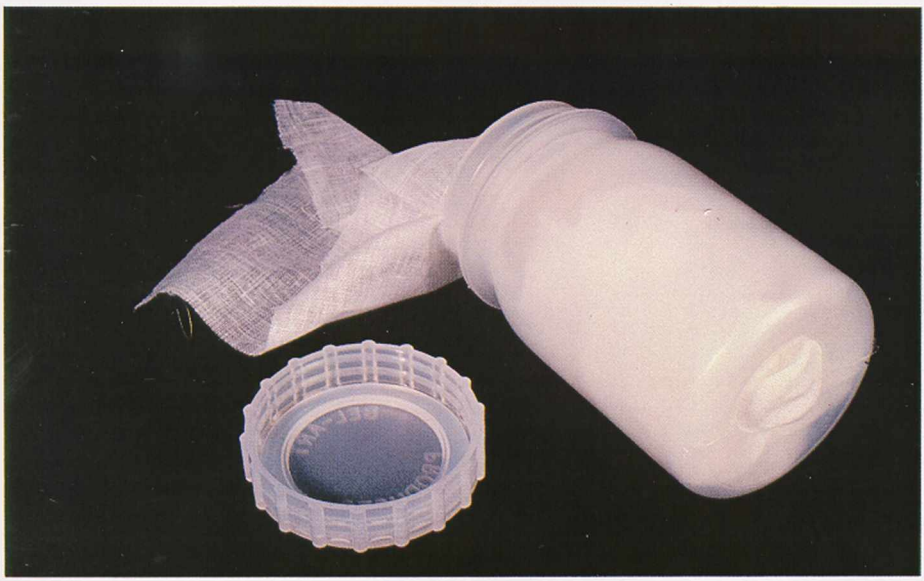

Figure V-3. The Spira swab is used for filtering large volumes of water through cotton gauze to recover *V. cholerae*.

date. Samples should be transported as quickly as possible to the laboratory in an ice chest with frozen refrigerant packs to prevent possible overheating (see Section A of this chapter for instructions for transport of specimens). If it will be longer than 3 hours between collection of swabs and arrival at the laboratory, the swabs should be placed in APW at the collection site before transport to ensure optimal recovery of *V. cholerae*. APW (300 to 500 ml) should be added to the specimens immediately upon arrival at the laboratory if it was not added at the site. The Moore swab and the associated sample water should make up approximately 10% to 20% of the total volume of the sample with APW added to obtain the optimal ratio of sample to enrichment broth for recovery. Incubate specimens at 35° to 37°C for 6 to 8 hours before plating, as described in Section F of this chapter.

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of the bottle is critical; if it is too large, the gauze is pulled out by the water as it flows through, and if it is too small the water passes through very slowly. Gauze 30 cm (12 in) wide is packed into the bottle in a layered fashion so that when water is poured into the top of the bottle it passes through the gauze and out of the bottle through the 2-cm hole in the bottom. The gauze should be properly layered to prevent the water from being channeled around the layers of gauze instead of being filtered. Enough gauze (approximately 4 to 6 ft or 120 to 180 cm) should be used to form a firm but still compressible pack and should fill about two-thirds of the bottle's volume. Sterilization of Spira swabs is optional. If swabs are to be sterilized, wrap each bottle in foil or brown paper and autoclave. Pour water to be sampled into the top and allow to drain out the bottom. Aseptically remove the gauze swab and place in flask or jar containing 100 ml of 10x APW. Add sufficient source water to final volume of 1 liter. Incubate at 35° to 37°C for 6 to 8 hours and plate to TCBS.

5. MPN Technique

The most probable number (MPN) method uses a multiple dilution-to-extinction approach for estimating microbial population. It is useful in situations where extremely low densities of organisms are encountered and where potential competitors complicate other enumeration methods. This method may be used to locate the source of contamination by establishing a gradient of concentrations of *V. cholerae* O1. Estimates of *V. cholerae* populations in water may be determined by an MPN procedure consisting of a 3-dilution, 3- or 5-tube replication series that uses enrichment in APW followed by plating to TCBS. However, if the water to be sampled is heavily contaminated with sewage, dilutions out to 10^{-6} may be necessary to reach an endpoint. If laboratory resources permit, a duplicate set of dilutions may be prepared to be incubated at 42°C. Methods for the MPN procedure are described in *Standard Methods for the Examination of Water and Wastewater*, published by the American Public Health Association.

E. Isolation of *V. cholerae* from Food, Sediment, and Other Environmental Samples

In addition to water, contaminated food can serve as a vehicle for the transmission of cholera. Foods commonly associated with cholera transmission have included fish (particularly shellfish harvested from contaminated waters), milk, cooked rice, lentils, potatoes, kidney beans, eggs, chicken, and vegetables. Freshly harvested oysters and fish are frequently cultured as sentinel specimens for surveillance purposes. The intestines, and to a lesser extent, the skin of freshly caught fish are more likely to harbor *V. cholerae* organisms than is the muscle tissue, but for fish in the market that have been scaled and cleaned, the flesh may be culture positive because of cross-contamination during cleaning and storage on ice.

Sediment, aquatic plants, plankton, and other environmental specimens may be sampled to monitor the incidence and ecology of *V. cholerae* O1 in various ecosystems and the importance of these as reservoirs in the transmission of cholera.

1. Preparation of food, sediment, and other environmental samples

Samples should be kept refrigerated (4°C) until cultured (see Section A of this chapter). Aseptically weigh a 25-g food sample into a sterile blender jar or stomacher bag (see Figure V-4). Cut large samples into smaller pieces before blending. Add a small amount of APW to the jar and blend thoroughly. After blending, add additional APW to bring the total amount added to 225 ml (10^{-1} dilution). For sediment and other environmental samples that do not require blending, weigh a 25-g sample and place into 225 ml of APW (10^{-1} dilution). If resources permit, prepare duplicate samples (for incubation at both 35° to 37°C and 42°C). Prepare two tenfold dilutions (10^{-2} and 10^{-3}) of the blended food samples or plankton/sediment samples in APW. If duplicate samples are prepared, both should be diluted. Dilutions may be made to 10^{-6} if enumeration of *V. cholerae* is desired. Refer to the *Bacteriological Analytical Manual* (U.S. Food and Drug Administration) for more information.

2. Preparation of oyster samples

To culture oysters, remove and combine the meat from 10 to 12 animals; include the shell liquor. Blend to mix. Blend 25 g of this composite with 225 ml of APW (10^{-1} dilution). Prepare two tenfold dilutions (10^{-2} and 10^{-3}) of the oysters in APW. If duplicate samples are prepared, both should be diluted. Because certain components of oyster meat are toxic to *V. cholerae* O1, process only a few oysters at a time and dilute the samples as quickly as possible. This process will dilute out competing microorganisms, as well as the bactericidal effects of the oyster meat on *V. cholerae*. A duplicate set of dilutions incubated at 42°C greatly enhances the chances of isolating *V. cholerae* and is strongly recommended when culturing oysters, even more than for other types of food specimens.

F. Incubation of APW

Incubate the APW (with caps loosened on all jars and dilution tubes) at 35° to 37°C for 6 to 8 hours. If duplicate dilutions are prepared, incubate the second set of jars and dilutions at 42°C for 6 to 8 hours. After incubation, streak to TCBS using one large or two smaller loopfuls from the surface and topmost portion of the broth, since vibrios preferentially migrate to this area. Do not shake or mix the tube before subculturing. If the APW cannot be subcultured after 6 to 8 hours of incubation, at 18 hours subculture to a fresh tube of APW. This second APW tube should be subcultured to TCBS following 6 to 8 hours of incubation. Incubate TCBS plates for 18 to 24 hours at 35° to 37°C.

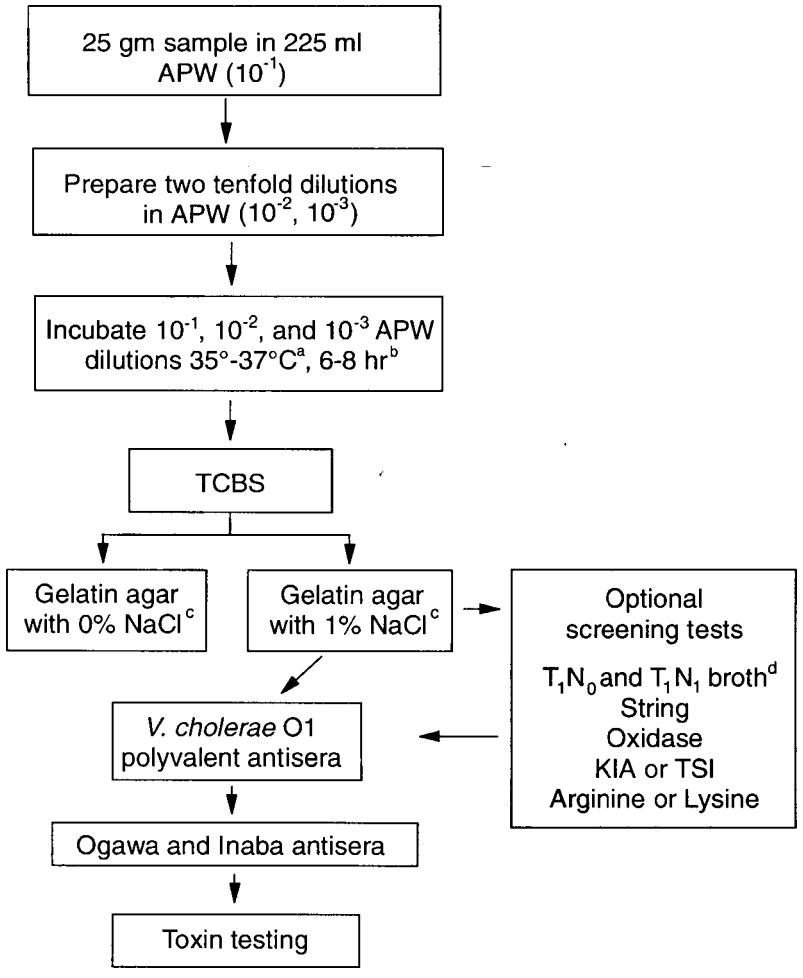

Figure V-4. Procedure for recovering *Vibrio cholerae* O1 from food or environmental specimens

[a] Duplicate APW dilutions may be prepared and incubated at 42°C.
[b] If the APW cannot be streaked after 6-8 hours of incubation, subculture at 18 hours to a fresh tube of APW; incubate for 6-8 hours, and then streak to TCBS.
[c] T_1N_0 and T_1N_1 agar may be used as an alternative to gelatin agar with 0% and 1% NaCl.
[d] Testing for growth in 0% and 1% NaCl may be done by subculturing from TCBS to a nonselective medium (e.g., gelatin agar with 1% NaCl, T_1N_1 agar, HIA) and then to T_1N_0 and T_1N_1 broth.

In some circumstances, it may be advantageous to incubate APW for 18 hours. For frozen food and oysters, incubate the homogenate in APW for 6 to 8 hours, then subculture an inoculum to TCBS. Reincubate the original homogenates for total incubation time of 18 hours, and replate the specimens to TCBS.

G. Isolation and Presumptive Identification

When culturing samples from an estuarine or marine environment, special methods must often be used to screen out the numerous other *Vibrio* species that grow well in APW and on TCBS agar. No single screening procedure for *V. cholerae* O1 is ideal for each laboratory and sample. The laboratorian should select a screening procedure based on available resources (for example, the availability of antisera), the competing organisms present in the environment being sampled, and the ability of the selective plating medium to inhibit the growth of those competing organisms. An effective procedure using a salt-free medium followed by biochemical screening and serologic tests is described below.

1. Salt-free media

Most of the *Vibrio* species other than *V. cholerae* that are frequently encountered in marine or estuarine specimens are halophilic or at least require a minimum amount of salt in culture media. For this reason, the ability of *V. cholerae* to grow on culture media with no added salt can be a useful selective characteristic.

Three or more colonies suspicious of *V. cholerae* may be subcultured from TCBS to two gelatin agar plates, one with 0% NaCl and the other with 1% NaCl (Figure V-4). See Chapter XI, "Preparation of Media and Reagents," for special instructions for preparation of gelatin agar. If the gelatinase reaction does not offer any advantage in the screening process, T_1N_0 (1% tryptone and 0% NaCl) agar and T_1N_1 (1% tryptone and 1% NaCl) agar may be used. Plates may be inoculated with as many as eight colonies by dividing them into eight sections. Growth should be inoculated in a straight line in the middle of each sector. Growth should be added to the gelatin plate without salt first since *V. cholerae* does not grow as well without salt and a larger inoculum may be required for that medium.

Since halophilic *Vibrio* spp. grow only on the medium with 1% NaCl, select isolates that grow on both the medium with 0% NaCl and medium with 1% NaCl. The gelatinase reaction (*V. cholerae* is positive) may be observed by holding the plate above a black surface or up to a light to observe the halo effect around the growth of gelatinase-positive organisms. Refrigeration of the gelatin agar plate for 15 to 30 minutes enhances the halo and makes it easier to observe this effect (see Chapter IV, "Isolation of *V. cholerae* from Fecal Specimens," for a description of gelatin agar). Growth from the agar medium with 1% NaCl may be tested directly in

V. cholerae O1 polyvalent antiserum or screened with the oxidase and string test before further biochemical testing.

An alternate procedure is to inoculate colonies suspicious of *V. cholerae* from TCBS to a nonselective medium (e.g., gelatin agar with 1% NaCl, T_1N_1 agar, HIA). Plates or tubed media may be used. Growth from this medium may then be inoculated into T_1N_0 and T_1N_1 broth to test for growth in the absence of salt before proceeding with further testing.

2. Biochemical screening tests

Slide serology is sufficient for a preliminary identification of *V. cholerae* O1 without further testing. However, it may be advantageous to screen with biochemical tests before testing with *V. cholerae* O1 antiserum if the TCBS medium used for isolation is not sufficiently selective to inhibit competitors such as *Aeromonas, Pseudomonas,* and *Enterobacteriaceae*; if those competitors are especially numerous in the environment sampled; or if the supply of antiserum is limited. Tests found to be useful for eliminating non-*V. cholerae* organisms are arginine (or arginine glucose slant), lysine (or lysine iron agar), string, oxidase, and Kligler iron agar (KIA) or triple sugar iron agar (TSI) (see Table IV-2 in Chapter IV, "Isolation of *V. cholerae* from Fecal Specimens"). Biochemical tests should be selected according to their ability to rule out the greatest number of competitors and will vary with different types of specimens. There is no need to use two biochemicals that rule out the same organism. For example, if arginine is used, it is not advantageous to also screen with lysine since they generally rule out the same organisms.

The string and oxidase tests may be performed on fresh growth from the gelatin with 1% NaCl agar plate (T_1N_1 or other nonselective medium), offering information immediately. The string test is useful for ruling out non-*Vibrio* spp., particularly *Aeromonas*. Arginine and lysine can be used to rule out *Aeromonas* and certain *Vibrio* spp. Arginine is generally more helpful than lysine, but lysine may be used if arginine is not available. KIA or TSI will rule out *Pseudomonas* and certain *Enterobacteriaceae* spp. Oxidase can be used to eliminate non-*Vibrio* spp., particularly the *Enterobacteriaceae*. (For descriptions of these tests see Chapter VI, "Laboratory Identification of *V. cholerae*")

Caps on all tubes of biochemicals should be loosened before incubation. This is particularly important for KIA or TSI slants since, if the caps are too tight and anaerobic conditions exist, the characteristic reactions of *V. cholerae* may not be exhibited.

3. Slide agglutination

Suspect *V. cholerae* isolates should be tested by slide agglutination with polyvalent *V. cholerae* O1 antiserum. Isolates that agglutinate in polyvalent antiserum may be reported as presumptive *V. cholerae* O1 but should be confirmed with agglutination in monovalent Ogawa and Inaba

antisera. Since nontoxigenic *V. cholerae* O1 is frequently encountered in environmental specimens (particularly marine and estuarine), all *V. cholerae* O1 isolates from environmental or food specimens should be tested for cholera toxin production after their identification has been confirmed. Isolates that are serologically confirmed to be *V. cholerae* O1 may be further characterized for hemolysis, biochemical identification, antimicrobial sensitivities, biotype, or molecular subtype. (Refer to Chapter VI, "Laboratory Identification of *V. cholerae,*" for a description of methods for serologic, biochemical, biotype, hemolysis, and sensitivity tests. Chapter VII describes methods for detection of cholera toxin production.)

References

1. Barrett TJ, Blake PA, Morris GK, Puhr ND, Bradford HB, Wells JG. Use of Moore swabs for isolating *V. cholerae* from sewage. J Clin Microbiol 1980;11:385-8.
2. Spira WM, Ahmed QS. Gauze filtration and enrichment procedures for recovery of *Vibrio cholerae* from contaminated waters. Appl Environ Microbiol 1981;42:730-3.
3. U.S. Food and Drug Administration. Bacteriological Analytical Manual, 7th ed. Arlington, Virginia: Association of Official Analytical Chemists, 1992.
4. American Public Health Association, American Water Works Association, and the Water Pollution Control Federation. Standard Methods for the Examination of Water and Wastewater, 17th ed. Washington, D.C.: American Public Health Association, 1989.

VI. Laboratory Identification of *Vibrio cholerae*

Members of the genus *Vibrio* are facultatively anaerobic, asporogenous, motile, curved or straight gram-negative rods. Vibrios either require NaCl or have their growth stimulated by its addition. All members of the genus *Vibrio,* with the exceptions of *V. metschnikovii* and *V. gazogenes,* are oxidase positive and reduce nitrates to nitrites. Within the *Vibrionaceae* are many different species, most of which are normal inhabitants of the aquatic environment. Of the more than 30 species within the *Vibrio–Photobacterium* complex, only 12 have been recognized as being pathogens for humans (Table VI-1). Although most of these 12 species are isolated from intestinal as well as extraintestinal infections, only *V. cholerae* is associated with epidemic cholera.

Unidentified vibrios have been called "marine species," or simply, "marine vibrios." These marine species are defined as *Vibrio* or *Photobacterium* strains that are oxidase positive, ferment D-glucose, do not grow in nutrient broth without added NaCl, but do grow in nutrient broth with added NaCl. Most organisms isolated from ocean or estuarian waters belong to the marine vibrio group and are difficult to identify except in a few specialized laboratories. Because they are not associated with human illness, marine vibrios need not be identified on a routine basis. Clinical and public health laboratories usually report the human pathogenic vibrios by genus and species and all other vibrios as "marine vibrio."

The minimum identification of *V. cholerae* O1 requires only serologic confirmation of the presence of O1 serotype antigens with suspect isolates. However, a more complete characterization of the organism may be necessary and may include various biochemical tests as well as the determination of other characteristics. The laboratory should decide when it is appropriate to perform these additional tests on clinical isolates, since they should not be a routine part of identification of *V. cholerae* O1. Generally, if the isolate is from a region that is threatened by epidemic cholera or is in the early stages of a cholera outbreak, it is appropriate to confirm the production of cholera toxin and the biochemical identification. Other tests that could provide important public health information include hemolysis, biotyping, molecular subtyping, and antimicrobial sensitivity assays. These tests should be performed on only a limited number of isolates. (See Chapter II, "The Role of the Public Health Laboratory.")

A. Serologic Identification of *V. cholerae* O1

The use of antisera is one of the most rapid and specific methods of identifying *V. cholerae* O1. Although identifying the serogroup and serotype of *V. cholerae* isolates is not necessary for treatment of cholera, this information may be of epidemiologic and public health importance (Table VI-2).

Table VI-1. Eight key differential tests to categorize the 12 clinically important *Vibrio* species into six groups (1)

Species	Growth in nutrient broth[a] 0% NaCl	Growth in nutrient broth[a] 1% NaCl	Oxidase	Nitrate to nitrite	Myo-inositol fermentation	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase
Group 1								
V. cholerae	+	+	+	+	-	-	+	+
V. mimicus	+	+	+	+	-	-	+	+
Group 2								
V. metschnikovii	-	+	-	-	V	V	V	-
Group 3								
V. cincinnatiensis	-	+	+	+	+	-	V	-
Group 4								
V. hollisae	-	+	+	+	-	-	-	-
Group 5								
V. damsela	-	+	+	+	-	+	V	-
V. fluvialis	-	+	+	+	-	+	-	-
V. furnissii	-	+	+	+	-	+	-	-
Group 6								
V. alginolyticus	-	+	+	+	-	-	+	V
V. parahaemolyticus	-	+	+	+	-	-	+	+
V. vulnificus	-	+	+	+	-	-	+	V
V. carchariae	-	+	+	+	-	-	+	-

Note: + = ≥90% positive; - = <10% positive; V = 10%–89% positive.

[a] Difco Laboratories, Detroit, MI.

Table VI-2. Characteristics of *Vibrio cholerae*

Classification method	Epidemic-associated	Not epidemic-associated
Serogroups	O1	Non-O1 (>130 exist)
Biotypes	Classical, El Tor	Biotypes not applicable to non-O1 strains
Serotypes	Inaba, Ogawa, Hikojima	These 3 serotypes not applicable to non-O1 strains
Toxin	Produce cholera toxin[a]	Usually do not produce cholera toxin; sometimes produce other toxins

[a] Nontoxigenic O1 strains exist, but are not epidemic-associated.

1. Serogroups of *V. cholerae*

Currently, there are more than 130 serogroups of *V. cholerae*, based on the presence of somatic O antigens. However, only the O1 serogroup is associated with epidemic and pandemic cholera. Other serogroups may be associated with severe diarrhea, but do not possess the epidemic potential of the O1 isolates and do not agglutinate in O1 antisera. Isolation of *V. cholerae* non-O1 from environmental sources in the absence of diarrheal cases is common. Laboratories may choose not to report the isolation of *V. cholerae* non-O1 when investigating cholera epidemics, since health care providers or public health officials may be unaware of the important epidemiologic differences between O1 and non-O1 isolates. The name "*Vibrio cholerae*" on a laboratory report may incorrectly imply that a non-O1 isolate is of epidemiologic importance. Confusion may be eliminated by reporting only whether *V. cholerae* serogroup O1 was or was not isolated.

2. Serotypes of *V. cholerae* O1

Isolates of the O1 serogroup of *V. cholerae* have been further divided into three serotypes, Inaba, Ogawa, and Hikojima (very rare). Serotype identification is based on agglutination in antisera to type-specific O antigens (see Table VI-3). Identifying these antigens is valid only with serogroup O1 isolates. For this reason, Inaba and Ogawa antisera should never be used with strains that are negative with polyvalent O1 antisera. Isolates that agglutinate weakly or slowly with serogroup O1 antisera but do not agglutinate with either Inaba or Ogawa antisera are not considered to be serogroup O1.

Table VI-3. Identifying characteristics of serotypes of *V. cholerae* serogroup O1

Serotype	Major O factors present	Agglutination in absorbed serum	
		Ogawa	Inaba
Ogawa	A, B	+	-
Inaba	A, C	-	+
Hikojima	A, B, C	+	+

Strains of one serotype frequently cross-react slowly and weakly in antiserum to the other serotype, depending on how well the serotype-specific antisera have been absorbed. Agglutination reactions with both Inaba and Ogawa antisera should be examined simultaneously, and the strongest and most rapid reaction should be used to identify the serotype. With adequately absorbed antisera, strains that agglutinate very strongly and equally with both the Ogawa and Inaba antisera are rarely, if ever, encountered. If such reactions are suspected, the strains should be referred to a reference laboratory for further examination and may be referred to as "possible serotype Hikojima."

3. Slide agglutination

Agglutination tests for *V. cholerae* somatic O antigens may be carried out in a petri dish or on a clean glass slide. An inoculating needle or loop, or sterile applicator stick, or tooth pick is used to remove a portion of the growth from the surface of a heart infusion agar (HIA), Kligler's iron agar (KIA), triple sugar iron agar (TSI), or other nonselective agar medium. Emulsify the growth in a small drop of physiological saline and mix thoroughly by tilting back and forth for about 30 seconds. Examine the suspension carefully to ensure that it is even and does not show clumping due to autoagglutination. If clumping occurs, the culture is termed "rough" and cannot be serotyped. If the suspension is smooth (turbid and free-flowing), add a small drop of antiserum to the suspension. Usually approximately equal volumes of antiserum and growth suspension are mixed, but the volume of suspension may be as much as double the volume of the antiserum. To conserve antiserum, volumes as small as 10 µl can be used. Mix the suspension and antiserum well and then tilt slide back and forth to observe for agglutination. If the reaction is positive, very strong clumping will appear within 30 seconds to 1 minute (Figure VI-1).

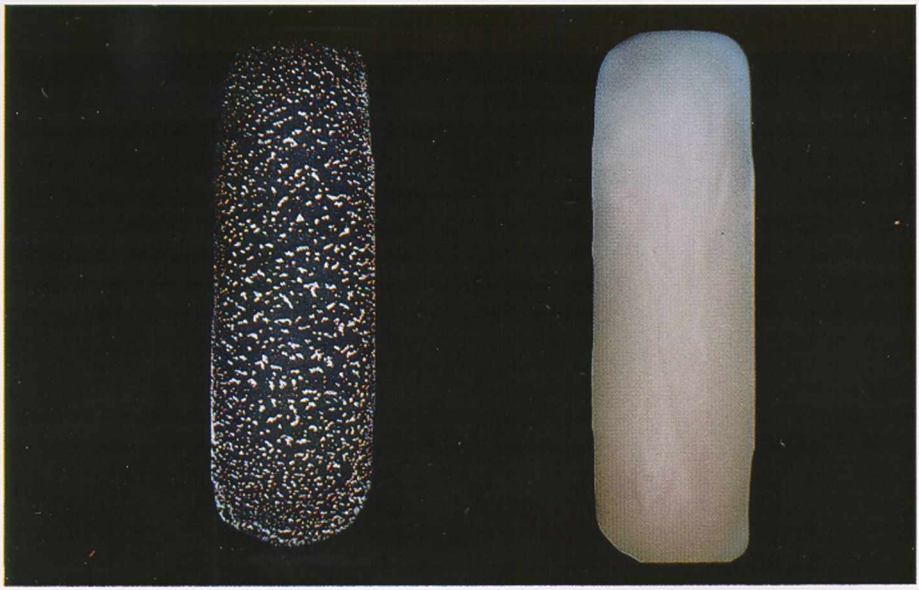

Figure VI-1. Antisera to the O1 serogroup of *V. cholerae* will agglutinate homologous organisms (left). A normal serum or saline control (right) does not show agglutination.

Table VI-4. Biochemical characteristics of typical isolates of *V. cholerae* O1

Test	% positive
Oxidase	100
String test	100
Kligler's iron agar	K/A, no gas, no H_2S
Triple sugar iron agar	A/A, no gas, no H_2S
Glucose[a] (acid production)	100
Glucose (gas production)	0
Sucrose (acid production)	100
Lysine[a]	99
Arginine[a]	0
Ornithine[a]	99
Growth in 0% NaCl[b]	100
Growth in 1% NaCl[b]	100
Voges-Proskauer[a]	75[c]

[a] Modified by the addition of 1% NaCl.

[b] Nutrient broth base (Difco Laboratories)

[c] Most isolates of *V. cholerae* serotype O1 biotype El Tor are positive in the VP test, whereas biotype classical strains are negative.

B. Biochemical Identification of *V. cholerae*

Since confirmation of *V. cholerae* O1 requires only identification of the O1 serotype antigens by slide agglutination, biochemical confirmation is only infrequently necessary (see Chapter II, "The Role of the Public Health Laboratory.") The tests listed in Table VI-4 constitute a short series of biochemicals that may be used to confirm isolates of *V. cholerae*. If the results of tests with an isolate are the same as those shown in Table VI-4, the identity of the isolate is confirmed as *V. cholerae*. However, if the isolate does not give results as shown in the table, additional tests will be necessary for identification. See Chapter XI, "Preparation of Media and Reagents," for instructions for preparing media and reagents for the biochemical tests shown in Table VI-4. The use of KIA or TSI, the oxidase and "string" tests, and arginine or lysine reactions may be helpful for screening isolates resembling *V. cholerae*. Screening procedures for fecal and environmental specimens are discussed in Chapters IV and V.

1. Oxidase test

Conduct the oxidase test with fresh growth from an HIA slant or any non-carbohydrate-containing medium. Do not use growth from thiosulfate citrate bile salts sucrose (TCBS) agar. Place 2 to 3 drops of oxidase reagent (1% tetramethyl-*p*-phenylenediamine) on a piece of filter paper in a petri dish. Smear the culture across the wet paper with a platinum (not nichrome) loop, a sterile wooden applicator stick, or toothpick. In a positive reaction, the bacterial growth becomes dark purple within 10 seconds (Figure VI-2). Color development after 10 seconds should be disregarded. Positive and negative controls should be tested at the same time. Organisms of the genera *Vibrio, Neisseria, Campylobacter, Aeromonas, Plesiomonas, Pseudomonas,* and *Alcaligenes* are all oxidase positive; all *Enterobacteriaceae* are oxidase negative.

2. String test

The string test may be performed on a glass microscope slide or plastic petri dish by suspending 18- to 24-hour growth from HIA or other noninhibitory medium in a drop of 0.5% aqueous solution of sodium deoxycholate. If the result is positive, the bacterial cells will be lysed by the sodium deoxycholate, the suspension will lose turbidity, and DNA will be released from the lysed cells causing the mixture to become viscous. A mucoid "string" is formed when an inoculating loop is drawn slowly away from the suspension (Figure VI-3). Most vibrios are positive, whereas *Aeromonas* strains are usually negative.

3. Kligler's iron agar or triple sugar iron agar

KIA and TSI are carbohydrate-containing screening media widely used in diagnostic microbiology. While similar in use, the two media vary in the carbohydrates they contain. The reaction of *V. cholerae* on KIA, which contains glucose and lactose, is similar to those of non-lactose-fer-

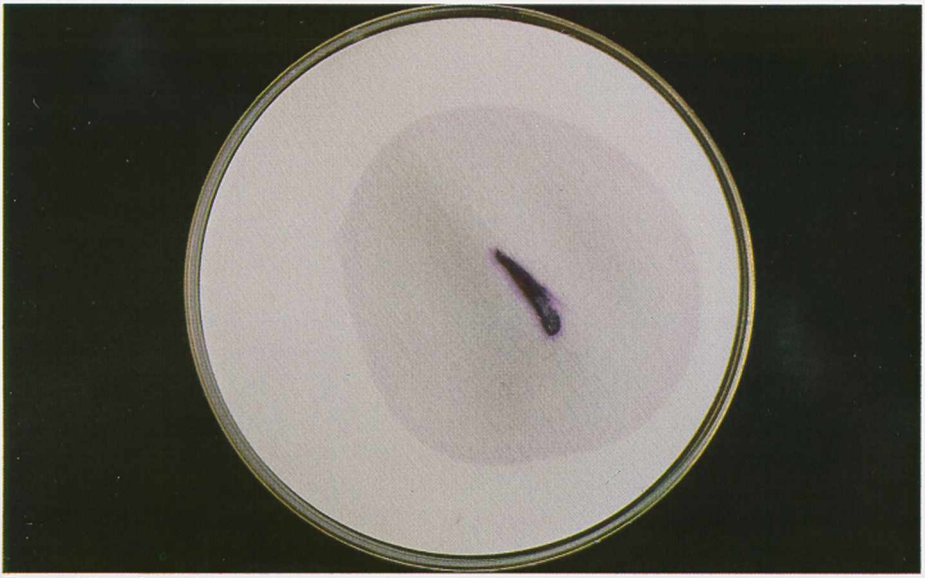

Figure VI-2. A positive oxidase test (as shown here) results in the development of a dark purple color within 10 seconds. *V. cholerae* is oxidase-positive, which differentiates it from oxidase-negative organisms such as the *Enterobacteriaceae*.

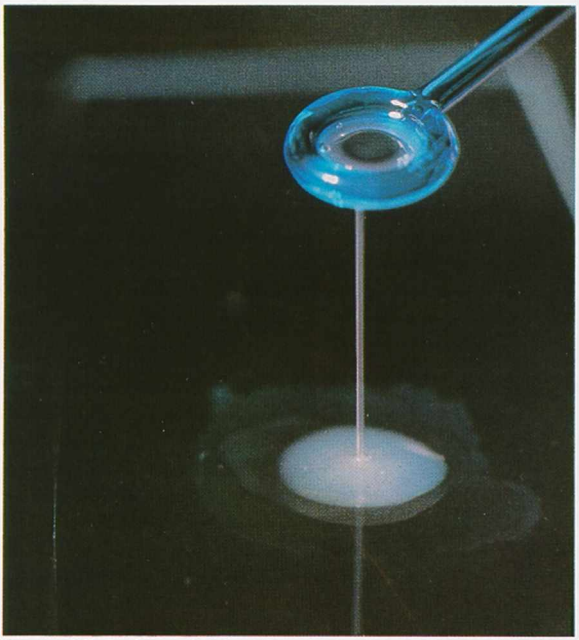

Figure VI-3. A positive string test, shown here with *V. cholerae*, is a rapid and simple method for distinguishing between the genus *Vibrio* (mostly positive) and *Aeromonas* (nearly always negative).

menting *Enterobacteriaceae* (K/A, no gas, no H_2S) (Figure VI-4). TSI, which contains sucrose in addition to glucose and lactose, gives reactions of A/A, no gas, and no H_2S. KIA or TSI slants are inoculated by stabbing the butt and streaking the surface of the medium. Slants should be incubated at 35° to 37°C and examined after 18 to 24 hours. Caps on all tubes of biochemicals should be loosened before incubation, but this is particularly important for KIA or TSI slants. If the caps are too tight and anaerobic conditions exist in the KIA or TSI tube, the characteristic reactions of *V. cholerae* may not be exhibited and an inappropriate reaction will occur.

4. Carbohydrates

Glucose and sucrose broths should be inoculated lightly from fresh growth. The broths should be incubated at 35° to 37°C and read at 24 hours. If fermentation tests are negative at 24 hours, they should be incubated for up to 7 days. Acid production is indicated by a pink color when Andrade indicator is used in the medium (Figure VI-5). *V. cholerae* ferments both glucose and sucrose but does not produce gas in either carbohydrate.

5. Decarboxylase/dihydrolase reactions

Arginine, lysine, ornithine, and control (without amino acid) broths modified by the addition of 1% NaCl should be inoculated lightly from a fresh culture. The broth in each tube should be overlayed with 4 to 5 mm of sterile mineral oil. Incubate at 35° to 37°C and read at 24 and 48 hours, but if the test is negative it should be incubated for up to 7 days. When bromcresol purple and cresol red are used as indicators, an alkaline (positive) reaction is purple, while a negative or acid reaction is indicated by a yellow color (Figure VI-6). The test is valid only if the control tube stays negative (yellow). *V. cholerae* is typically positive for lysine decarboxylase, while certain other *Vibrio* spp. are negative and *Aeromonas* spp. are variable. *V. cholerae* is typically negative for arginine dihydrolase, while *Aeromonas*, *Plesiomonas*, and certain other *Vibrio* spp. are positive. *V. cholerae* is positive for ornithine decorboxylase.

Lysine iron agar and arginine glucose slant

A lysine iron agar (LIA) slant may be used instead of lysine broth (above) to test for the production of lysine decarboxylase; similarly, an arginine glucose slant (AGS; U.S. Food and Drug Administration. Bacteriological Analytical Manual, 6th ed. Arlington, Virginia: Association of Official Analytical Chemists, 1992) may be used instead of arginine broth to test for the production of arginine dihydrolase. These slants are used most frequently as part of a screening procedure (see Chapters IV and V). LIA and AGS should be inoculated by stabbing the butt and streaking the slant. Organisms that produce lysine decarboxylase in LIA (or arginine dihydrolase in AGS) cause an alkaline reaction (purple color) throughout the medium. Organisms without these enzymes typically produce an alka-

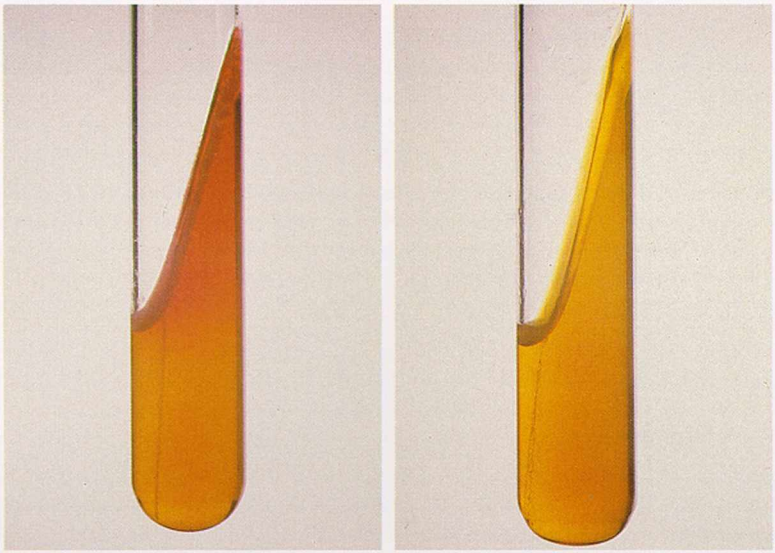

Figure VI-4. Reactions of *V. cholerae* in Kligler's iron agar (left) and triple sugar iron agar (right).

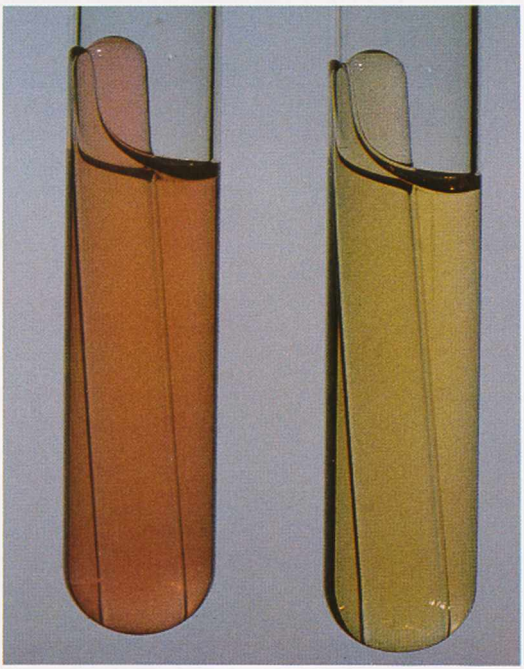

Figure VI-5. With Andrade indicator in the carbohydrate medium, a pink color develops if fermentation has occurred, while negative reactions appear yellow.

line slant (purple) and an acid butt (yellow). *V. cholerae* gives a K/K reaction in LIA (lysine positive) but produces a K/A reaction (arginine negative) in AGS.

6. Salt broths

The 0% and 1% salt broths (nutrient broth base [Difco Laboratories, Detroit, Michigan]; see Chapter XI, "Preparation of Media and Reagents," for special instructions for preparation of salt test broths) should be inoculated very lightly from fresh growth. The inoculum should be light enough to prevent visible turbidity before incubation of the broths. The broths are incubated at 35° to 37°C and read at 18 to 24 hours. In the absence of overnight growth, they may be incubated for up to 7 days (Figure VI-7).

7. Voges-Proskauer test

The CDC reference laboratory uses a modification of the Voges Proskauer test procedure that increases its sensitivity with the vibrios. In this modification, the test medium (MR-VP broth) incorporates 1% NaCl, reagent A consists of 5% alpha-naphthol in absolute ethanol, and reagent B is a solution of 0.3% creatine in 40% KOH (potassium hydroxide). The test organism is incubated in MR-VP broth for 48 hours before reagents A and B are added. A cherry red color indicates a positive reaction (Figure VI-8).

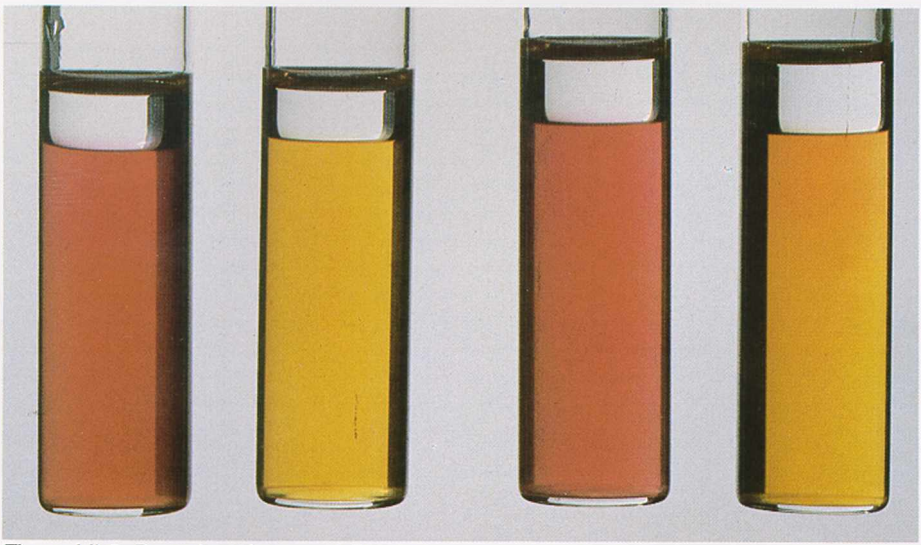

Figure VI-6. Decarboxylase and dihydrolase reactions for *V. cholerae* are, from left to right, lysine (+), arginine (-), ornithine (+), and control (-).

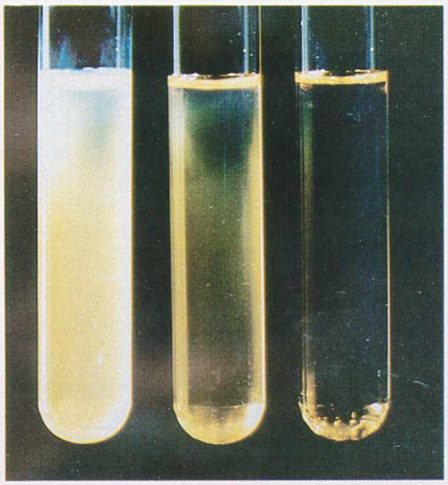

Figure VI-7. *V. cholerae* grows in the absence of NaCl (tube B), but growth is stimulated by the addition of 1% NaCl (tube A). Tube C, 0% NaCl, inoculated with *V. parahaemolyticus*, shows no growth.

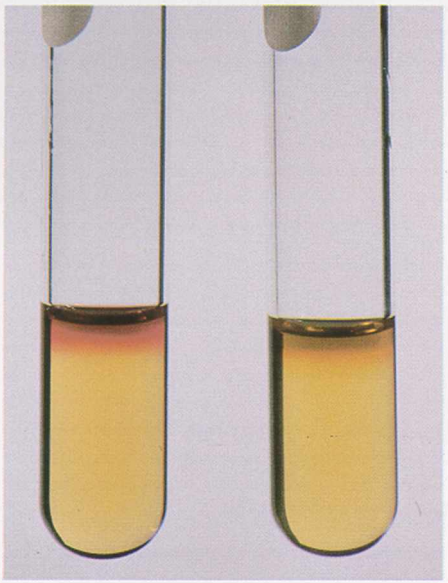

Figure VI-8. *V. cholerae* produces acetoin, which is detected in the Voges-Proskauer test, giving a red (positive) reaction (left). A negative reaction is on the right.

8. Susceptibility to vibriostatic compound O/129

Susceptibility to 2,4-diamino-6,7-diisopropyl-pteridine phosphate (referred to as O/129 or vibriostatic compound) has been recommended and used as a primary means for differentiating between *Vibrio* (which are sensitive to O/129) and *Aeromonas* (resistant to O/129). While most isolates of *V. cholerae* have been sensitive to O/129, several recent reports have described clinical and environmental isolates that were resistant to this compound. In these reports *V. cholerae* O1 and non-O1 isolates were resistant to 10 and 150 μg of O/129, thus resembling *Aeromonas*. Caution should be exercised when relying on this test.

C. Hemolysis Testing

Historically, the classical and El Tor biotypes were differentiated by the ability of the El Tor group to lyse erythrocytes. However, by 1972 almost all El Tor isolates worldwide were nonhemolytic. The two exceptions to this trend have been the U.S. Gulf Coast and the Australia clones of *V. cholerae* O1, which are strongly hemolytic when assayed by either the plate or tube hemolysis assay (Table VI-5). For this reason, hemolysis continues to be a useful phenotypic characteristic for differentiating the Gulf Coast and Australia clones of *V. cholerae* O1 from El Tor strains from the rest of the world, including Latin America.

1. Plate hemolysis

Blood agar plates containing 5% to 10% sheep blood should be streaked to obtain isolated colonies. Plates should be incubated at 35° to 37°C for 18 to 24 hours. Hemolytic colonies should have clear zones around them where red blood cells have been totally lysed, and a suspected hemolytic strain should be compared with a strongly hemolytic con-

Table VI-5. Hemolytic activity of *V. cholerae* O1 classical and El Tor biotypes

Biotype/location	Hemolytic activity
Classical	Negative
El Tor/Australia	Strongly positive
El Tor/US Gulf Coast	Strongly positive
El Tor/Latin America	Negative
El Tor/Asia, Africa, Europe, Pacific[a]	Negative

[a] Strains isolated between 1963 and 1992.

trol strain (Figure VI-9). Strains that give incomplete hemolysis (incomplete clearing of the red blood cells) should not be reported as hemolytic.

On aerobic sheep blood agar plates, nonhemolytic *V. cholerae* frequently produces greenish clearing around areas of heavy growth but not around well-isolated colonies. This phenomenon, often described as "hemodigestion," is produced by metabolic by-products which are inhibited by anaerobic incubation of the blood agar plate. For this reason, when aerobic growth conditions are used, hemolysis should be determined around isolated colonies, not in areas of confluent growth. Also, aerobic blood agar plates should be incubated for no more than 18 to 24 hours, since the hemodigestion effect is accentuated during longer incubation periods.

Aerobic incubation of the plate for no longer than 24 hours, although not optimal for detection of hemolysis, will permit differentiation of strongly hemolytic strains, such as the U.S. Gulf Coast and Australia clones, from the nonhemolytic Latin American strains. If the results of the plate hemolysis assay are not conclusive, test the strain by the tube hemolysis method, which is less susceptible to misinterpretation than the plate method.

2. Tube hemolysis assay

Controls: Use two well-characterized strains of *V. cholerae*. One should be strongly hemolytic, the other nonhemolytic.

1) Wash 20 ml of sheep erythrocytes in 25 ml of phosphate-buffered saline (PBS), 0.01 M, pH 6.8–7.2. Repeat twice for a total of 3 washes. Prepare a 1% (vol/vol) suspension of packed sheep erythrocytes in PBS.
2) From fresh growth, inoculate test strains and controls into heart infusion broth (or Trypticase soy broth) with 1% glycerol (pH 7.4) and incubate at 35° to 37°C for 24 hours. After incubation, centrifuge to sediment bacterial cells; remove the supernatants with a Pasteur pipette.
3) Divide the supernatants into two equal portions. One aliquot is heated to 56°C for 30 minutes. Make serial twofold dilutions of both the heated and unheated supernatants in PBS (dilute to 1:1,024).
4) Add 0.5 ml of the 1% suspension of sheep erythrocytes in PBS to 0.5 ml of each dilution of supernatant.
5) Incubate in a water bath at 37°C for 2 hours. Remove the suspensions from the water bath, and hold overnight at 4°C.
6) Examine for evidence of hemolysis. Nonhemolyzed red blood cells will settle to the bottom of the test tube and form a "button" (Figure VI-10). No button will be present if the cells are lysed by hemolysin. Hemolysin titers should be recorded as the highest dilution at which complete hemolysis has occurred.

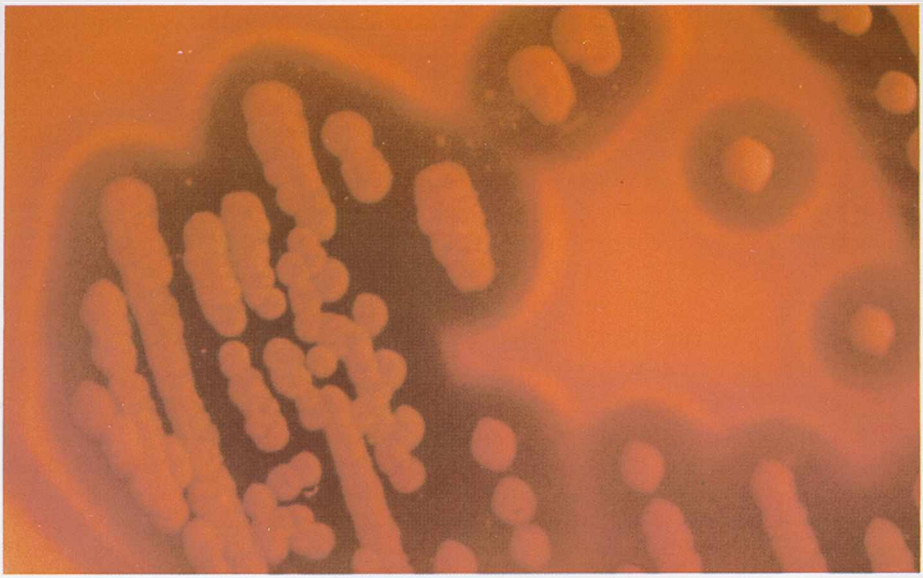

Figure VI-9. A hemolytic strain of *V. cholerae* on a sheep blood agar plate.

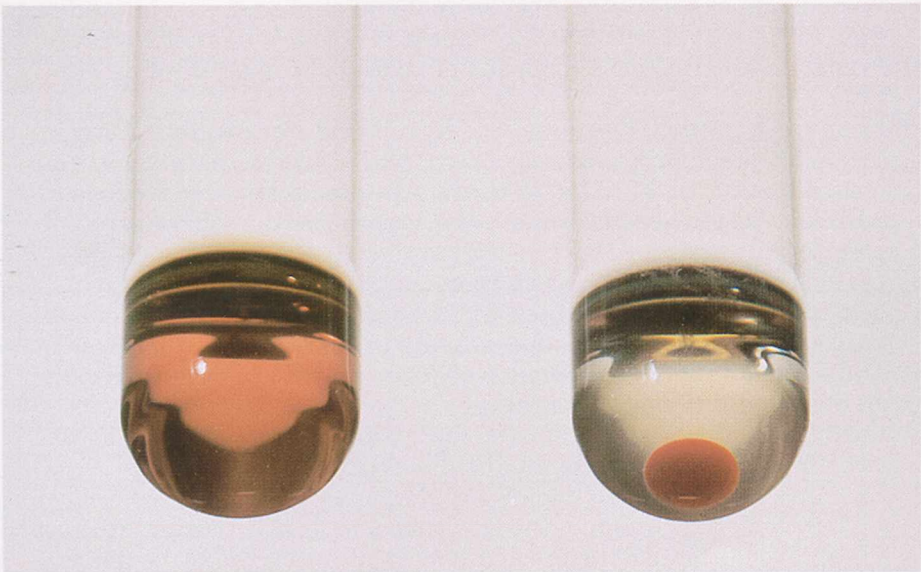

Figure VI-10. Tube hemolysis, shown in the tube on the left, is demonstrated by the absence of a "button" of sedimented cells and the presence of free hemoglobin in the supernatant.

Table VI-6. Differentiation of classical and El Tor biotypes of *V. cholerae* serogroup O1

Property	Reaction	
	Classical	El Tor
Voges-Proskauer (modified, with 1% NaCl)	-	+
Zone around polymyxin B (50 U)	+	-
Agglutination of chicken erythrocytes	-	+
Lysis by bacteriophage:		
Classical IV	+	-
El Tor V	-	+

7) Compare results of the heated and unheated supernatants. Heated tubes should show no hemolysis, since the hemolysin of *V. cholerae* is heat-labile, and if present, is inactivated by the 56°C incubation (step 3, above). Titers of 2 to 8 are considered intermediate, and titers of 16 or above are strongly positive.

D. Tests for Determining Biotypes of *V. cholerae* O1

The differentiation of *V. cholerae* O1 into classical and El Tor biotypes is not necessary for control or treatment of patients, but may be of public health or epidemiologic importance in helping identify the source of the infection, particularly when cholera is first isolated in a country or geographic area. Only limited numbers of carefully selected isolates should be biotyped. Biotyping is not appropriate for *V. cholerae* non-O1, and the tests can give atypical results for nontoxigenic *V. cholerae* O1. The tests shown in Table VI-6 are commonly used in determining the biotype of *V. cholerae* O1. At least two or more of these tests should be used to determine biotype, since results can vary for individual isolates.

The El Tor biotype is currently predominant throughout the world and is the only biotype that has been isolated in the Western Hemisphere. The classical biotype is seen only rarely in most places, with the exception of Bangladesh.

1. Voges-Proskauer test

The Voges-Proskauer test has been used to differentiate between the El Tor and classical biotypes of *V. cholerae* O1. Classical biotypes usually give negative results; El Tor isolates are generally positive.

2. Polymyxin B sensitivity

Sensitivity to the antimicrobial agent polymyxin B has been used to differentiate the biotypes of *V. cholerae* O1 (see Section E in this chapter for a description of antimicrobial susceptibility testing procedures). A 50-unit polymyxin B disk (Mast Diagnostics, Merseyside, U.K.) is used for this test, and known strains of classical and El Tor biotypes are used as controls. The El Tor biotype is usually resistant to this concentration of polymyxin B and will not give a zone of inhibition (Figure VI-11). If there is any doubt about the result of this test, other biotyping tests should be performed or the isolate should be sent to a reference laboratory for confirmation. Classical strains are usually sensitive to polymyxin B and will give a zone of inhibition. Because the zone size is not important, any zone is interpreted as a positive result.

3. Hemagglutination (direct test)

Fresh chicken or sheep red blood cells can be used for this assay. A 2.5% (vol/vol) suspension of washed (3 times) and packed (by centrifugation) cells is made in normal saline after the final wash. A large loopful of the red cell suspension is placed on a glass slide. A small portion of the growth from a nonselective agar slant is added to the red cells with a needle or loop and is mixed well. In a positive test, agglutination of the red cells occurs within 30 to 60 seconds (Figure VI-12). Hemagglutinating (El Tor) and nonhemagglutinating (classical) control strains should be used with each new suspension of red cells. Strains of classical *V. cholerae* O1 that have aged in the laboratory or have undergone repeated passage in broth may cause hemagglutination and should not be used as controls.

4. Bacteriophage susceptibility

Biotype may be determined by the susceptibility of an isolate of *V. cholerae* serogroup O1 to a specific bacteriophage. Classical strains of *V. cholerae* O1 are sensitive to cholera bacteriophage "Classical IV"; El Tor isolates are susceptible to bacteriophage "El Tor 5." Although these tests are very reliable, the propagation, storage, and use of bacteriophage is technically demanding and is usually performed in only a few reference laboratories. If biotype determination by bacteriophage susceptibility is needed, it should be performed by a laboratory that uses this method routinely.

The use of bacteriophage in the biotyping of *V. cholerae* O1 is briefly described as follows. The isolate to be tested is grown overnight in pure culture on a noninhibitory medium. From the overnight growth, brain heart infusion broth (or Trypticase soy broth) is inoculated and incubated for 6 hours at 35° to 37°C. A lawn of bacteria in log-phase growth (OD = 0.1) is then seeded onto the surface of a brain heart infusion agar plate by dipping a cotton swab into the 6-hour broth and lightly inoculating (swabbing) the entire surface of the plate. Positive and negative control strains

Figure VI-11. The El Tor strain of *V. cholerae* O1 on the left is resistant to the action of the antimicrobial agent polymyxin B (50 unit disk). A classical strain of *V. cholerae* O1, on the right, shows a characteristic zone of inhibition.

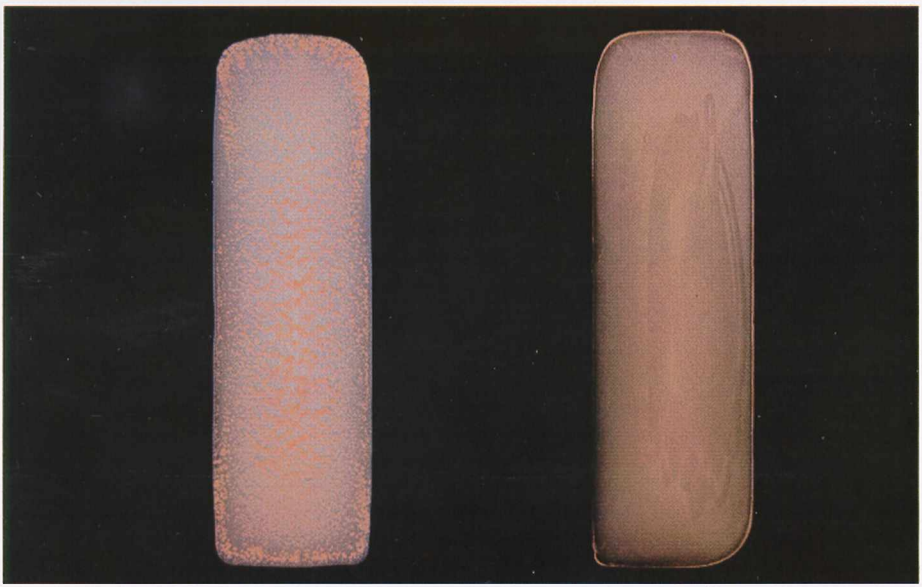

Figure VI-12. *V. cholerae* O1 biotype El Tor agglutinates sheep red blood cells as shown on the left. The classical biotype of *V. cholerae* O1 on the right does not agglutinate red blood cells.

should also be included. A drop of the bacteriophage diluted to routine test dilution (a measure of concentration of active bacteriophage particles) is applied to the bacterial lawn. The plate is incubated overnight and read the next day. If the bacteria are susceptible to the bacteriophage, they will be lysed, and there will be a zone of lysis in the bacterial lawn.

E. Antimicrobial Susceptibility Test (Agar Disk Diffusion Method)

Because antimicrobial resistance has been a growing problem in some parts of the world, the susceptibility of *V. cholerae* O1 strains to antimicrobial agents should be monitored periodically. Although the disk diffusion technique is the most commonly used method for antimicrobial susceptibility testing, interpretive criteria have not been established for *V. cholerae* O1, and the method's reliability for this organism is unknown. To ensure the accuracy of susceptibility results for *V. cholerae* O1, agar or broth dilution methods should be used. Refer to the Manual of Clinical Microbiology (6) for a description of these procedures. If a laboratory cannot routinely perform one of the dilution techniques, the disk diffusion method may be used to screen for antimicrobial resistance. Table VI-7 lists the interpretive criteria for the antimicrobial agents that are currently recommended by the World Health Organization for treatment of cholera (tetracycline, doxycycline, furazolidone, trimethoprim-sulfamethoxazole, erythromycin, and chloramphenicol) as well as some other commonly used antimicrobial drugs. These criteria, which have been standardized for the *Enterobacteriaceae*, may be used as tentative zone size standards for screening for antimicrobial resistances in *V. cholerae* O1 until interpretive criteria have been validated. When the disk diffusion method is used for screening, any isolates that fall within the intermediate or resistant ranges should be tested with a dilution method to obtain the minimum inhibitory concentration of the drug in question.

Procedure for agar disk diffusion

For a more complete description of this method, refer to the Manual of Clinical Microbiology (4).

Mueller Hinton agar medium should be prepared and autoclaved according to the manufacturer's directions. After the agar has been cooled to approximately 50°C in a water bath, the medium should be poured into 15 x 150-mm petri dishes to a depth of 4 mm (approximately 60 to 70 ml per plate). Dry the plates in an incubator for 10 to 30 minutes before use.

Prepare a 0.5 McFarland turbidity standard by adding 0.5 ml of 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 1% sulfuric acid. The turbidity standard should be in a test tube identical to the one to be used to grow the test organism in broth. The McFarland standard should be sealed with wax, Parafilm, or some other means to prevent evaporation, and may be stored for up to 6 months at room temperature (22° to 25°C) in the dark.

Table VI-7. Zone size interpretative standards for the *Enterobacteriaceae* for selected antimicrobial disks (not validated for *V. cholerae* O1)

Antimicrobial agent	Disk potency (μg)	Zone diameter (mm)[a]			Zone diameter limits (mm) for *E. coli* ATCC 25922
		Resistant	Intermediate	Sensitive	
Chloramphenicol	30	≤12	13-17	≥18	21-27
Doxycycline	30	≤12	13-15	≥16	18-24
Erythromycin	15	≤13	14-22	≥23	8-14[b]
Furazolidone	100	≤13	14-17	≥18	22-26[c]
Trimethoprim-sulfamethoxazole	1.25/23.75	≤10	11-15	≥16	24-32
Tetracycline	30	≤14	15-18	≥19	18-25
Ciprofloxacin	5	≤15	16-20	≥21	30-40
Nalidixic acid	30	≤13	14-18	≥19	22-28

[a] Source: National Committee on Clinical Laboratory Standards (NCCLS), 1992. Zone sizes for *V. cholerae* have not been established by NCCLS.

[b] Source: World Health Organization.

[c] Source: Manufacturer.

Each culture to be tested should be streaked onto a noninhibitory agar medium (blood agar, brain heart infusion agar, or Trypticase soy agar) to obtain isolated colonies. After incubation at 35° to 37°C overnight, select well-isolated colonies with an inoculating needle or loop and transfer the growth to a tube of sterile broth (Mueller-Hinton broth, heart infusion broth, or Trypticase soy broth). Emulsify a sufficient quantity of bacterial growth in the broth so that the turbidity approximates that of the 0.5 McFarland standard. This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn. If necessary, turbidity can be reduced by adding sterile broth. Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile nontoxic swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium 3 times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum. Finally, swab all around the edge of the agar surface.

The working supply of antimicrobial disks should be kept in the refrigerator. After removal from the refrigerator, the containers should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation that occurs when warm air reaches the cold container. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before using. The antimicrobial disks should be applied to the plates as soon as possible, but no longer than 15 minutes after inoculation. After the disks are placed on the plate, the plate should be placed in an incubator at 35°C for 16 to 18 hours.

After overnight incubation, the diameter of the zones of complete inhibition (including the diameter of the disk) is measured and recorded in millimeters. The measurements can be made with a ruler on the undersurface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. The zones of growth inhibition should be compared with the zone-size interpretative table (see Table VI-7), and recorded as susceptible, intermediate, or resistant to each drug tested.

Susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. To obtain reliable test results, it is important to include control organisms with each test and to follow the procedure precisely (see Table VI-7 for diameters of the zones of inhibition for the quality control strain). A decrease in potency of the disks after storage may be indicated by a decrease in the size of the inhibition zone around the control strain.

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VII. Detection of Cholera Toxin

A. Mode of Action of Cholera Toxin

The production of cholera toxin (CT) is an essential virulence property of epidemic strains of *Vibrio cholerae* O1. Each CT molecule is composed of five B (binding) subunits and one A (active) subunit. The B subunits bind to G_{M1} ganglioside receptors on epithelial cells of the intestinal mucosa. After attachment, cleavage occurs between the A subunit and the A2 component, facilitating entry of the A1 component into the cell. The A1 component stimulates the production of the enzyme adenylate cyclase, which is responsible for the production of cyclic AMP (cAMP). Increased intracellular levels of cAMP result in a disruption of the active transport of electrolytes across the cell membrane, which hinders fluid absorption and leads to fluid secretion into the small intestine. When the volume of the fluid entering the colon from the small intestine is greater than its reabsorptive capability, diarrhea occurs. CT is very similar to *Escherichia coli* heat-labile enterotoxin (LT), both antigenically and in mechanism of action; therefore, most of the toxin assays for detection of CT are also applicable to LT, and vice-versa.

B. Indications for Testing for CT Production

The value of routine CT testing in a diagnostic laboratory varies with the epidemiology of cholera in a specific country or community. During an outbreak of cholera, the isolation of *V. cholerae* possessing the O1 antigen from symptomatic patients correlates well with toxin production and virulence, and there is no need to routinely test isolates for CT. This is also true in most endemic cholera situations with a reasonably high frequency of disease. However, in endemic disease settings where the incidence of cholera is low or in the early stages of an outbreak, most *V. cholerae* O1 strains isolated from diarrheal stool should be tested for toxin. (See Chapter II, "The Role of the Public Health Laboratory," for a discussion of when it is necessary to test isolates for cholera toxin production.) Since nontoxigenic *V. cholerae* O1 strains are occasionally encountered in environmental specimens (particularly marine and estuarine waters), all food or environmental *V. cholerae* O1 isolates should be tested for cholera toxin production after the identification has been confirmed.

Before testing for toxin, the identity of isolates as *V. cholerae* O1 should be confirmed. Non-O1 *V. cholerae* strains may produce CT or other toxins such as heat-stable enterotoxin or Shiga-like toxin, but these strains are very rare and have not been associated with epidemic disease. Therefore, there seems to be little public health benefit in testing sporadic isolates of non-O1 *V. cholerae* for CT or other possible toxins.

Although both clinical and public health needs warrant at least some CT assays, those needs are usually most efficiently and economically met

at the reference laboratory level. Laboratories should select the most appropriate method for their needs and capabilities.

C. Historical Overview of CT Assay Methods

There are several approaches to assaying for cholera toxin, including tests for toxin activity, toxin antigens, and toxin-coding genes. The selection of a specific assay depends on the training, experience, and facilities available to the laboratory. Table VII-1 summarizes important characteristics of some of the more common assays used for detecting cholera toxin.

1. Bioassays

Animal methods

In the early 1950s, investigators discovered that injection of enterotoxin preparations into ligated segments of intestine (ileal loops) of rabbits (and later other animals including pigs, dogs, and calves) caused accumulation of fluid. This discovery resulted in the development of the first cholera enterotoxin assay, the adult rabbit ileal loop, which before the 1970s was the most widely used assay for CT. This model has been used extensively to study the mechanisms of action of CT, *E. coli* LT, and other enterotoxins. After exteriorization and ligation of the rabbit's small intestine, a cell-free supernatant is injected into each ileal loop and the abdomen is closed for 18 hours. The rabbit is then euthanized, the intestine removed, and the loops measured and weighed to determine the amount of fluid accumulation stimulated by the toxin. Results are expressed as volume of fluid per length of intestinal loop. This test is not only excessively stressful for the animals but is also time-consuming, cumbersome, and difficult to standardize. The test is relatively expensive in terms of numbers of animals required, since only about 8 to 14 supernatants may be tested per animal, not including positive and negative controls; also, each set of supernatants must be done in duplicate animals, and the orientation of supernatants must be reversed from one animal to the next.

The infant rabbit infection model was developed in 1955 and can be used for assay of both *V. cholerae* and *E. coli* enterotoxins. Seven-day-old infant rabbits are infected with the test organism by gastric intubation or by direct intragastric or intralumenal (small intestine) injection. The animals are observed for watery diarrhea and eventual death due to dehydration. Alternatively, after a 7-hour incubation period, the animals are killed; the intestines are removed and the fluid volume is measured per centimeter of intestine. The drawbacks to the infant rabbit method are the variability of results and the expense of using one animal for every isolate to be tested.

Table VII-1. Commonly used methods for detection of cholera toxin

Assay	Sensitivity (per ml)	Type of assay	Specific target of assay	Sample tested
Rabbit ileal loop	30 ng	Bioassay	Stimulation of fluid accumulation	Culture supernatant
Infant rabbit assay	250-500 ng	Bioassay	Stimulation of fluid accumulation	Broth culture or supernatant
Rabbit skin test	0.1-3.5 ng	Bioassay	Permeability factor	Culture supernatant
Y1 mouse adrenal cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
Chinese hamster ovary cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
G_{M1}-ELISA	10 pg	Immune	B subunit[b]	Culture supernatant
Coagglutination	50 ng[a]	Immune	B subunit[b]	Culture lysates
Reverse passive latex agglutination	1-2 ng	Immune	B subunit[b]	Culture supernatant
DNA probe	Detects *ctx* gene	Genetic	*ctx* gene	DNA (colony blot)
PCR	Detects *ctx* gene	Genetic	*ctx* gene	DNA (crude cell lysate)

[a] Sensitivity for detection of cholera toxin using antiserum to *E. coli* heat-labile enterotoxin.

[b] B-subunit of cholera toxin molecule.

The rabbit skin test, or vascular permeability factor assay, has been used to detect either CT or LT activity, with the specificity of the assay determined by the neutralization of activity by a standardized amount of antisera against CT. A cell-free culture supernatant of *V. cholerae* or *E. coli* and dilutions of antisera are injected intradermally into the shaved back of a young adult rabbit. Approximately 30 to 40 supernatants may be tested per rabbit. This is followed 18 hours later by an intravenous injection of Evans blue dye. CT-mediated increased capillary permeability leads to perfusion of the dye in the skin (bluing reaction), with localized induration at the injection sites. The area of "bluing" is measured relative to a negative control. This procedure permits the assay of 30 to 40 cultures per rabbit and is thus more economical in terms of numbers of animals required than other animal systems such as the infant rabbit and ileal loop assays.

Tissue culture methods

Tissue culture methods are very sensitive and reproducible and have been used extensively to assay for toxin production. The action of toxin on cells in culture has allowed successful investigation of the molecular basis of pathogenicity. In addition, these assay systems can be used to detect CT-neutralizing antibody. However, tissue culture methods require skilled workers and special reagents and equipment. These are techniques best suited for use in laboratories with existing tissue culture facilities.

The Y1 mouse adrenal (Y1) and Chinese hamster ovary cell cultures have been the standard tests for detecting CT and LT, although other cell lines, including Vero monkey kidney cells, are also sensitive. The CT or LT, if present in the bacterial culture supernatants added to the cells, stimulates the production of adenylate cyclase, which elevates the intracellular concentration of cAMP. The increased amounts of cAMP result in a morphologic response that can be seen under the microscope (CHO cells elongate and Y1 cells become rounded). Positive reactions may be confirmed by neutralization of the toxic effects in cell culture with antiserum to CT (or LT) or with ganglioside G_{M1}, which is the receptor site for CT and LT in host cell membranes and thus binds either of these toxins. For the toxin assay, a suspension of Y-1 cells is dispensed into 96-well microtiter plates. Usually, one 75-cm^2 flask of Y-1 cells is sufficient to seed up to 25 microtiter plates. Each microtiter plate may be used to test as many as 60 supernatants (30 supernatants if performed in duplicate) if the outside wells of the plate are not used.

2. Immunoassays

ELISA

CT is highly immunogenic for humans and laboratory animals. As a result, many immunologic techniques have been developed for detecting

CT. The discovery that G_{M1} ganglioside is the natural receptor for CT and LT and its subsequent purification led to the development of a ganglioside-capture enzyme-linked immunosorbent assay (G_{M1}-ELISA) for the detection of these toxins. To perform the G_{M1}-ELISA, culture supernatants are added to microtiter plate wells coated with G_{M1} ganglioside. Toxin bound to the G_{M1} receptors is then detected by adding antiserum to CT, followed by enzyme-conjugated antiglobulin antibody. Instead of using G_{M1} to coat the plate, a second antibody may be used to bind CT. If 96-well microtiter plates are used for the toxin assay, 30 supernatants may be tested in duplicate per microtiter plate (the outside wells are not usually used to test supernatants).

Coagglutination

Because many strains of *Staphylococcus aureus* have an outer coat of protein A that can directly combine with IgG, serologic reagents have been prepared in which a specific IgG antibody is adsorbed onto the surface of staphylococcal cells for use in an agglutination reaction. The test reagent is relatively inexpensive to prepare but requires a specific anti-CT (or anti-LT) antibody. Coagglutination for *E. coli* LT is rapid and simple to perform and requires little specialized laboratory equipment or training of laboratory personnel; however, this test has never been used to detect CT from culture supernatants or lysates of *V. cholerae* O1.

Latex agglutination

The latex agglutination test uses specific anti-CT or anti-LT bound to latex particles. The latex agglutination technique requires high quality antiserum or purified antibodies, a suitable latex preparation, and readily available laboratory supplies. A commercial version of this test is available in kit form and is described later in this chapter.

3. DNA-based Assays

DNA probes

Molecular tests that identify pathogenic microorganisms based on DNA sequences unique to the pathogen have many applications in diagnostic and public health microbiology. Specific DNA sequences within the cholera toxin gene(s) have been used as probes to detect homologous DNA sequences in toxigenic *V. cholerae* isolates. In practice, a variety of DNA molecules can be used as probes, including cloned DNA, restriction fragments of cloned DNA, polymerase chain reaction-generated DNA amplicons, and oligonucleotides. The probe is first labeled with an easily detectable molecule, such as a radioisotope, an enzyme, or a ligand, and is then hybridized to DNA from the test organism. The probe hybridizes only to those organisms containing homologous sequences. Nonhybridized probe is washed away, and the remaining hybridized probe is detected in a specific assay.

Polymerase chain reaction (PCR)

A second approach to molecular diagnostic tests that are based on specific DNA sequences is PCR. In PCR, the enzyme DNA polymerase is used to synthesize or amplify multiple copies of a specific DNA sequence (amplicon), which can then be detected on an agarose gel or with DNA probes. The DNA amplicon is defined by the location of two short, specific DNA oligonucleotides that bracket the sequence of interest and are used as primers by the DNA polymerase. Like DNA probes, the target for a PCR test is a virulence gene or DNA sequence that is unique to a pathogen. The toxigenicity of a *V. cholerae* isolate can be tested using PCR and primers that specifically amplify only CT genes. PCR has the advantages of being a very rapid technique that does not require pure cultures or even viable organisms. Some PCR tests can amplify DNA segments directly from stool, food, or environmental specimens; thus, the presence of an organism in a specimen can be determined without culturing the organism. Also, PCR has applications in other molecular techniques. It can be used to rapidly produce labeled DNA probes for restriction fragment length polymorphism (RFLP) analysis and colony blots.

The sensitivity and specificity of DNA-based assays provide an advantage over conventional methods. The use of DNA probes or PCR avoids the difficulties encountered with *V. cholerae* strains that do not express CT at detectable levels but possess *ctx* genes. The use of nonradioactive DNA labels, such as biotin and digoxigenin, has eliminated the technical problems associated with radioisotopic labels. While DNA-based diagnostic tests require specific training in molecular methods and more expensive reagents, the advantages of simplicity, sensitivity, safety, and stability have made DNA-based techniques invaluable to research and clinical laboratories.

D. Production of CT for Laboratory Assays

Optimal growth conditions for *V. cholerae* do not always correspond with optimal CT production in vitro. The optimal conditions for CT production vary according to the medium used. For this reason, specialized media have been developed for production of CT. In general, an incubation temperature of 30°C has been found to be superior to 37°C for toxin production with *V. cholerae* of either biotype. With El Tor strains, using Craig's medium, the best combination of time and temperature is 30°C for 48 hours without aeration. For classical biotype strains, 30°C with vigorous shaking for 48 hours provides the best yield of toxin. If 37°C incubation temperatures must be used, AKI medium is recommended. Although AKI allows the production of CT at 37°C, it has the disadvantage of having a short shelf life and must be prepared weekly. *V. cholerae* El Tor grown in AKI medium should be incubated at 37°C for 20 hours without shaking. AKI medium has not been evaluated for toxin production by classical biotype strains.

CT is actively exported by *V. cholerae* into the culture medium, unlike *E. coli* LT, which is usually found in the periplasmic space. Antimicrobial agents such as polymyxin B or lincomycin, which enhance LT accumulation in media, have no effect on the release of CT. It is therefore unnecessary to use either drug in media for production of CT.

Most cholera toxin assays, both immunologic methods and bioassays, test for CT in culture supernatants. Assays for toxin activity require intact CT, whereas some antigenic assays only detect the B subunit of CT and do not require complete toxin. The growth conditions for CT production described below are recommended for optimal expression of complete (active) CT, as required by rabbit ileal loop, rabbit skin test, and tissue culture assays.

Production of cholera toxin

1) Streak cultures to be tested on a nonselective slant (such as heart infusion agar). Incubate overnight at 35°C to 37°C. Include four control strains (two positive and two negative).
2) Inoculate strains into 5 ml of Craig's medium in 16 x 125-mm screw cap tubes. Keeping caps loose, incubate at 30°C for 48 hours without shaking. [Note: cultures may be tested after only 24 hours' incubation, but 48 hours is optimal for CT production.]
3) Centrifuge to sediment the bacterial cells and draw off supernatant with a Pasteur pipette. Store supernatant at 4°C until ready to test. Freeze at -70°C if the supernatant is to be to be stored for longer than 7 days.

E. Y-1 Assay for CT

The Y-1 clone of mouse adrenal tumor cells is sensitive to concentrations of CT as low as 10 picograms per milliliter. However, *Vibrio* and other bacterial genera may produce extracellular heat-labile products that cause nonspecific rounding, which may be misinterpreted as being caused by CT. It is preferable to neutralize positive or doubtful reactions with specific antisera to the toxin or other specific binding substances such as G_{M1} ganglioside, the natural receptor for the toxin in cell membranes.

Materials

- CO_2 incubator set at 37°C, 5.0% CO_2
- Laminar flow hood
- Inverted phase-contrast microscope
- Sterile conical centrifuge tubes
- Centrifuge
- Sterile tissue culture flasks (75 cm^2 growth area)

- Sterile, flat-bottom, 96-well microtiter plates for tissue culture
- Ham's F-10 Nutrient Mixture (GIBCO Laboratories, Grand Island, N.Y.) with 15% horse serum, 2.5% fetal calf serum, and gentamicin (10 μg/ml)
- 0.2% trypsin

Weekly procedures for tissue culture assay and maintenance

Y-1 adrenal cells are maintained in monolayer culture in Ham's F-10 nutrient mixture. Routinely, tissue culture flasks with a 75-cm^2 growth area are filled with 25 ml of medium and incubated at 37°C in a humidified 5% CO_2 atmosphere. All cell manipulations are done in a laminar flow hood, and cells are examined by using an inverted phase-contrast microscope.

Day 1

1) Pour off the F-10 medium from the confluent monolayer (1 week of growth is usually required). Wash the cell monolayer with 5 ml sterile phosphate-buffered saline (PBS) and pour off.
2) Add 1.5 ml of 0.2% trypsin to the flask, and leave the trypsin-covered monolayer at room temperature until cells begin to loosen from the plastic surface (5 to 10 minutes).
3) Add 5 ml of F-10 medium to the flask to neutralize the trypsin. (The Ham's F-10 is stored at 4°C, and should be brought to 37°C in a water bath before all medium changes and cell subcultures). If any monolayer remains, scrape it from the flask surface with a sterile rubber scraper.
4) Transfer the suspended cells (approximately 6.5 ml) to a sterile conical centrifuge tube and centrifuge at 500 to 1000 x *g* for 5 minutes.
5) Suction off the supernatant leaving a sediment of Y-1 adrenal cells in the centrifuge tube. Resuspend the cells in 5 ml of fresh F-10 medium with a Pasteur pipette.
6) Using a Pasteur pipette, dispense 6 drops of the cell suspension into each flask, to which has been added 25 ml fresh F-10 medium. As a general rule, duplicate flasks are seeded and carried.
7) For the toxin assay, which is run in flat-bottom, 96-well microtiter plates, make a 1:50 to 1:100 dilution of the cell suspension. Dispense the suspension, approximately 0.15 ml per well. Therefore, one flask usually can seed 12 to 25 plates. Stack the plates and cover the top plate to prevent contamination and evaporation.
8) Place flasks (with loose caps) and/or microtiter plates in the CO_2 incubator.

Day 2

Check flasks and/or wells under the microscope for cell growth.

Day 3 (late afternoon)

Using a Pasteur pipette, inoculate each well of the microtiter plate containing a monolayer of Y-1 adrenal cells, with 2 drops (50 µl) of the toxin supernatants (including positive and negative controls). (See Section D for methods for growing *V. cholerae* cultures for CT production.) Be sure the microtiter plates and appropriate record sheets are coded before the actual transfer. Restack microtiter plates, and incubate in CO_2 at 37°C overnight.

Day 4 (morning)

Read assay results. Examine wells at 100x or 200x magnification, using an inverted stage phase microscope. Compare test wells with positive control wells. CT and LT cause rounding of Y-1 adrenal cells (Figure VII-1). For the CT or LT assay, a positive well contains more than 10% rounded Y-1 cells. Occasionally, cytotoxic activity in the supernatant will result in dead, lysed, or detached cells, which may mask the rounding effect of CT. If this occurs, dilution of the supernatant (in an attempt to dilute out the cytotoxin) may allow the rounded cells to be visualized.

Method for neutralization of cholera enterotoxin

Refer to Section D in this chapter for methods for growing *V. cholerae* cultures for CT production. Dilute test supernatants 1:4 by using PBS (pH 7.2) containing 0.1% gelatin (PBS/G). If available, pure cholera toxin preparation should be used as a positive control. Use high-titer antiserum to cholera toxin in this assay (at CDC the anti-CT antiserum used for the

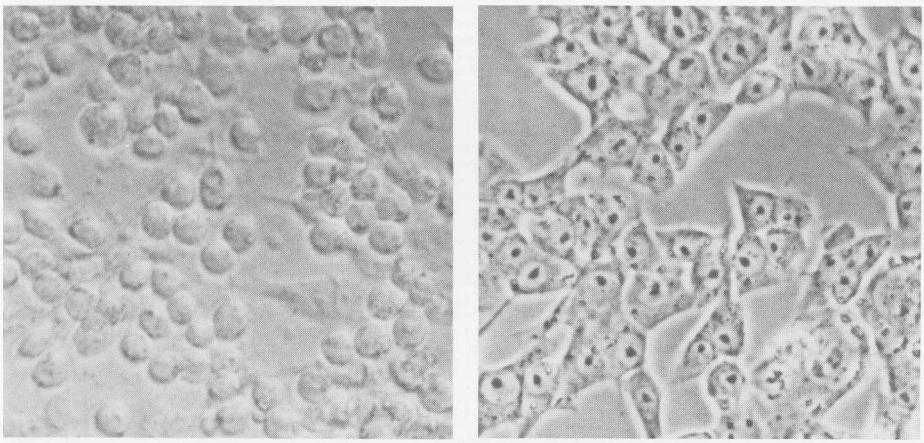

Figure VII-1. The panel on the left shows typical rounding of Y-1 mouse adrenal cells caused by the presence of cholera toxin. Normal Y-1 cells are shown in the panel on the right.

G_{M1}-ELISA is also used for neutralization in Y1 cells). Dilute the antiserum in PBS/G.

Prepare twofold dilutions of the anti-CT antiserum in PBS/G starting at 1:10 and ending at 1:10,240. Mix equal volumes of the antiserum dilutions with undiluted supernatant and the 1:4 dilution of the supernatant. Repeat the procedure for the rest of the supernatants and purified CT. Incubate the supernatant with antiserum mixtures in a water bath at 37°C. After 1 hour, transfer 50 µl of each supernatant with antiserum mixture in duplicate to the Y1 monolayer in the microtiter plate. Add non-neutralized supernatant for each test culture to one well for use as a control. Incubate cells overnight, and read to determine the highest dilution of anti-CT that neutralizes the rounding effect of the CT.

F. G_{M1}-ELISA for CT

The G_{M1}-ELISA is a sensitive immunoassay for the detection of CT. It uses an enzyme-labeled immunoglobulin, which is quantified by measuring its activity on a specific substrate. Microtiter wells are coated with ganglioside G_{M1}, the natural receptor for CT (Figure VII-2). Alternatively, microtiter wells may be coated with antibody to CT, but the coating antibody must be prepared in a different animal species than the second anti-CT antibody used in the assay. Culture supernatants are then added to the coated wells. CT molecules in the supernatant bind to the G_{M1} in the well and react with the anti-CT antibody. Antibody to the specific animal species' immunoglobulin conjugated to an enzyme (alkaline phosphatase or horseradish peroxidase) is added and reacts with the well-bound anti-CT antibody. Finally, the enzyme substrate is added, and

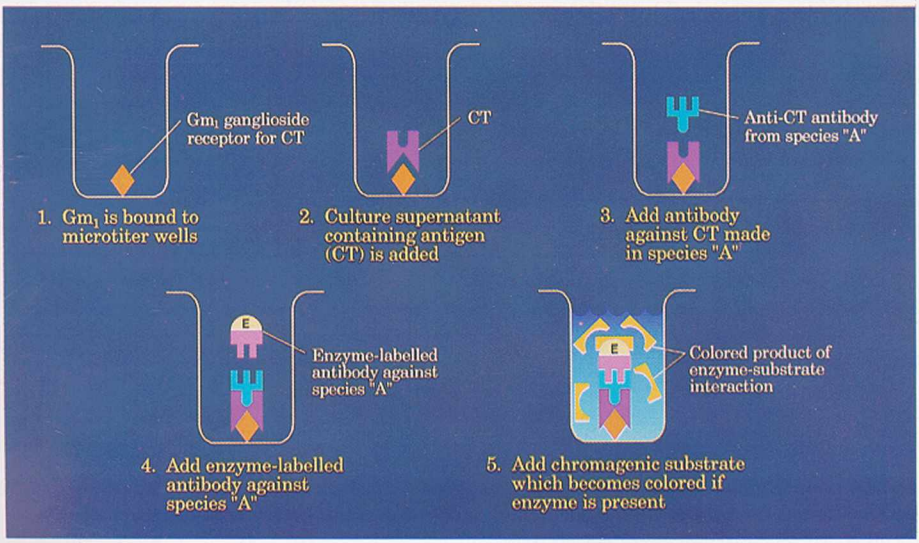

Figure VII-2. Schematic diagram of the G_{M1}-ELISA for the detection of cholera toxin.

the bound enzyme degrades the substrate, forming a colored product that indicates the presence of CT, LT, or other immunologically related molecules. These reactions can be read spectrophotometrically or visually. A summary of the ELISA method is presented in Table VII-2. All materials and reagents for the G_{M1}-ELISA for CT are individually available from commercial sources, including antiserum to CT.

Equipment

- Polystyrene or polyvinyl microtiter plates, flat-bottom or round-bottom
- Micropipettes and multichannel pipettes
- ELISA plate reader (optional)

Reagents

(See Chapter XI, "Preparation of Media and Reagents," for instructions for preparing the following reagents.)

- G_{M1} ganglioside (Sigma Chemical Co., St. Louis, Mo.)
- PBS with and without 0.05% Tween 20 and bovine serum albumin
- Goat antibody to CT_b (Calbiochem Corp., La Jolla, Calif.) [Note: As an alternative to goat anti-CT antibody, antibody produced in another animal species or monoclonal anti-CT antibody can be used.]
- Anti-goat (or whatever species was used to produce the anti-CT antibody) globulin labeled with alkaline phosphatase or horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.).
- *p*-nitrophenyl phosphate (Sigma Chemical Co.) dissolved in diethanolamine buffer [Note: Several substrates are available for horseradish peroxidase, each with specific requirements for preparation.]

  The optimal dilution of each specific reagent preparation should be determined by titration. A sample titration procedure is described in this section.

Controls

At least two known *V. cholerae* positive control and two known negative control supernatants should be included on each microtiter plate.

Performance of the test

1) Add 100 µl of G_{M1} diluted appropriately in PBS to the inner 60 wells of the microtiter plate. Fill empty wells around the perimeter of the plate with PBS/Tween. Cover with a plate sealer or place the plate in a moist chamber and let it stand at room temperature overnight or at 35° to 37°C for 4 hours.

Table VII-2. ELISA for detection of cholera toxin

Step	Reagent	Diluent	Concentration	Volume/well	Incubation time
Coating	G_{M1}	PBS	2 μg/ml[a]	100 μl	Overnight
Blocking	BSA	PBS	1%	150 μl	30 minutes
Sample	Culture supernatant	None	Undiluted	100 μl	60 minutes
Blocking	BSA	PBS	1%	150 μl	30 minutes
Antibody	Antibody to CT prepared in goat	PBS with 0.1% BSA	1:2000[a]	100 μl	60 minutes
Conjugate	Alkaline phosphatase-labeled anti-goat IgG	PBS with 0.1% BSA	1:500[a]	100 μl	60 minutes
Substrate	*p*-nitrophenyl phosphate	Diethanolamine buffer	1 mg/ml	100 μl	10-20 minutes
Stop reaction	NaOH	H_2O	3 M	50 μl	None

[a] The optimal dilution of each reagent should be determined by titration.

2) Wash the plate three times with PBS/Tween as follows: Invert the plate and gently tap out the contents onto an absorbent towel. With a wash bottle or another suitable dispensing device, fill each well with PBS/Tween (approximately 200 µl). Let the plate stand for 3 minutes. Remove the PBS/Tween and repeat twice. To store the plate for later use, leave the third wash in the plates and refrigerate (4°C). The plate may be stored for 4 to 6 weeks.
3) Block the remaining binding sites by filling each well with 150 µl of PBS with 1% BSA. Incubate the plate at room temperature for 30 minutes. Remove the contents and wash the wells three times with PBS/Tween as described in step 2.
4) With a micropipette, add 100 µl of each supernatant to duplicate wells in the plate. Leave a single row of wells around the perimeter of the plate empty. This will allow 30 tests per plate. Fill empty wells around the perimeter of the plate with PBS/Tween. Place the plate in a moist chamber or seal it and incubate it for 1 hour at 35° to 37°C. Wash plates three times as described in step 2.
5) Add 150 µl of PBS with 1% BSA to each of the wells, and incubate at room temperature for 30 minutes. Wash three times (step 2).
6) Add 100 µl of goat anti-CT_b serum (diluted in PBS containing 0.1% BSA) to each of the test wells. Place the plate in a moist chamber or seal it and incubate it at 35° to 37°C for 1 hour. Wash plate three times.
7) Add to each test well 100 µl of alkaline phosphatase-labeled rabbit anti-goat globulin diluted appropriately in PBS containing 0.1% BSA. Place the plate in a moist chamber or seal it and incubate it at 35° to 37°C for 1 hour. Wash plate three times.
8) Add 100 µl of the enzyme substrate solution (*p*-nitrophenyl phosphate in diethanolamine buffer) to all of the wells. Incubate the plate at room temperature until color development in the positive control wells reaches a suitable intensity but not so long that excessive color develops in the negative wells (approximately 10 to 20 minutes, but no longer than 30 minutes).
9) Stop the reaction by adding 50 µl of 3 M NaOH to each well. Mix well.

Reading the test results

1) Compare the positive control wells with the negative control supernatants. There should be little or no color in the negative control wells. In the positive control wells, a distinct yellow color should be visible (Figure VII-3).

2) Compare the amount of color in the test wells with the color in the negative control wells. Specimens that clearly develop a stronger color than the negative controls are considered positive.
3) If a microtiter plate reader is used, the wavelength should be set to the appropriate setting for the substrate used. For the *p*-nitrophenyl phosphate, the wavelength should be set to 405 nm. A positive to negative (P/N) ratio is calculated by dividing the optical density (OD) of the unknown sample by the mean OD of the wells containing the negative controls. Samples with a P/N ratio of 2.0 or greater are considered positive.

Titration of reagents for G_{M1}-ELISA for CT

1) Dilute G_{M1} in PBS to concentrations listed below:

   0.5 µg/ml
   1.0 µg/ml
   2.0 µg/ml
   5.0 µg/ml

2) Add 100 µl of G_{M1} ganglioside (Sigma Chemical Co.) to each of the inner 60 wells of polyvinyl round-bottom microtiter plates or polystyrene, flat-bottom plates containing the following G_{M1} dilutions:

Plate 1	0.5 µg/ml
Plate 2	1.0 µg/ml
Plate 3	2.0 µg/ml
Plate 4	5.0 µg/ml

Cover with a plate sealer or place the plates in a moist chamber and let them stand at room temperature overnight. Wash plates three times with 200 µl of PBS/Tween.

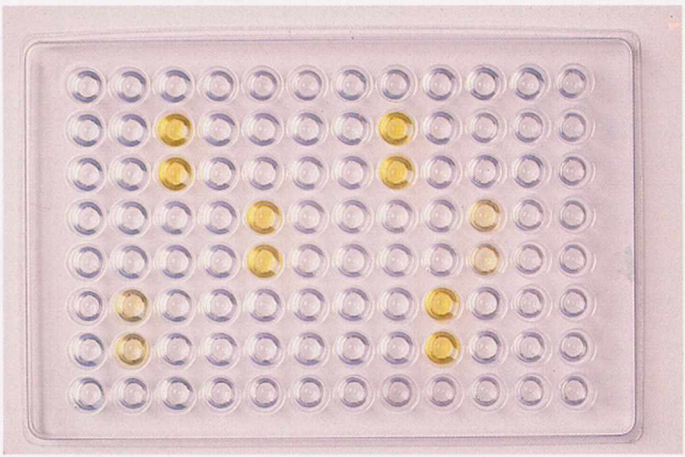

Figure VII-3. In the G_{M1}-ELISA for CT, a positive reaction is indicated by the development of a distinct yellow color in the wells of the microtiter plate.

3) To block the remaining binding sites of the wells, fill each well with 150 µl of PBS with 1% BSA and incubate the plate at room temperature for 30 minutes. After incubation, remove the contents and wash the wells three times with 200 µl of PBS/Tween.

4) Rehydrate pure CT (Calbiochem Corp.) according to manufacturer's instructions. Dilute in PBS with 0.1% BSA as follows (at least 4 ml of each dilution is needed for titration on four plates):

Dilution	**CT concentration**
10^{-3}	1.0 µg/ml
10^{-4}	100.0 ng/ml
10^{-5}	10.0 ng/ml
10^{-6}	1.0 ng/ml
10^{-7}	100.0 pg/ml
10^{-8}	10.0 pg/ml

Add to each plate 100 µl of each different dilution of CT so that each dilution takes up one row on the plate (rows B, C, D, E, F, G). Fill empty wells with PBS/Tween. Incubate at 35°C to 37°C for 1 hour. Wash plates three times with 200 µl of PBS/Tween.

5) Block with 150 µl of 1% BSA. Incubate at room temperature for 30 minutes. Wash the plate three times.

6) Prepare five dilutions of anti-CT in PBS with 0.1% BSA:

1:500
1:1000
1:2000
1:5000
1:10000

Note: monoclonal antibody should be tested at dilutions 1:100, 1:200, 1:500, 1:1000.

Add 100 µl of diluted anti-CT to each plate so that each dilution takes up one column of the plate (columns 2-6). Repeat for columns 7-11. Fill empty wells with PBS/Tween. Incubate at 35° to 37°C for 1 hour. Wash the plate three times.

7) Dilute alkaline phosphatase-labeled conjugate in PBS with 0.1% BSA as follows:

1:500
1:1000

Add 100 µl of each conjugate dilution to each plate so that each dilution takes up half the plate (columns 2-6 for one dilution and columns 7-11 for the other dilution). Fill empty wells with PBS/Tween. Incubate at 35°C to 37°C for 1 hour. Wash the plate three times.

8) Add 100 µl of *p*-nitrophenyl phosphate substrate. Incubate for 30 minutes at room temperature.

9) Add 50 µl of 3 M NaOH to stop the reaction, and read the results spectrophotometrically or visually. Note the highest dilution of each reagent which provides suitable color intensity. The reagent that is the most difficult to obtain or the most expensive should be used in the highest dilution possible.

G. Latex Agglutination Assay for CT

The VET-RPLA kit (Oxoid Limited, Hampshire, England) is designed for the detection of CT or LT in culture supernatant fluids. This test procedure is known as reversed passive latex agglutination (RPLA). Polystyrene latex particles are sensitized with purified antiserum produced in rabbits immunized with purified *V. cholerae* enterotoxin. These latex particles will agglutinate in the presence of CT or LT. A control reagent, which consists of latex particles coated or "sensitized" with nonimmune rabbit globulins, is provided.

The test is performed in V- or U-bottom microtiter plates (V-bottom plates are preferred; flat-bottom plates are not suitable for this procedure). Dilutions of culture supernatant to be tested are made in two columns of wells. [Note: test supernatants should be prepared according to instructions in Section D of this chapter; do not prepare supernatants by the method described in the instructions included with the kit because *V. cholerae* does not usually produce CT in sufficient quantities in alkaline peptone water (APW).] A suspension of antibody-coated latex particles is added to the first column, and unsensitized control latex is added to the second column. If sufficient amounts of either CT or LT are present in the supernatant, agglutination will occur. Agglutination results from the formation of cross-linkages among the latex particles bound to CT molecules. This lattice-like structure will settle and form a diffuse layer at the bottom of the well. If enterotoxin is absent or at a concentration below the detection level, no lattice structure will be formed, and a "button" of unagglutinated latex particles will form on settling.

Materials required

- 96-well plates (V-bottom or U-bottom) with lid
- Fixed or adjustable micropipette and tips (25 µl)
- Sensitized latex (latex suspension sensitized with specific antibodies [rabbit IgG] against cholera toxin, provided with kit).
- Control latex (latex suspension coated with nonimmune rabbit globulins, provided with kit).
- Control cholera toxin (dehydrated, provided with kit)
- Diluent: PBS containing BSA (provided with kit)

Performance of the test

1) Each sample requires two columns (eight wells per column) on the plate. With a pipette or dropper, dispense 25 µl of diluent in each well except the first well in each column.
2) Add 25 µl of test sample to the first and second well of each set of two columns.
3) Using a pipette or diluter and starting at the second well of each column, remove 25 µl and perform twofold dilutions. Stop at the seventh well in the column so that the eighth well contains diluent only. Discard the extra 25 µl from the seventh well.
4) Add 25 µl of sensitized latex to each well of the first column for each test and control supernatant. Add 25 µl of unsensitized control latex to each well of the second column for each test and control supernatant.
5) Mix the contents of each well by agitating the microtiter plate gently by hand.
6) To avoid evaporation, cover the plate with a lid and place in a plastic storage container along with a wet paper towel. Leave the plate undisturbed on a vibration-free surface at room temperature for 20 to 24 hours.

Reading the test results

1) Examine each well in each column against a black background for agglutination. The presence of either CT or LT will cause agglutination, demonstrated by the formation of a lattice structure, which upon settling forms a diffuse layer on the bottom of the well (Figure VII-4). If the enterotoxins are absent or at a concentration below the

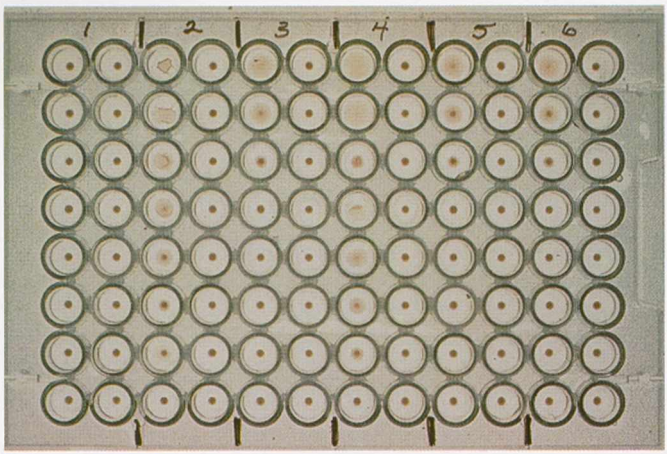

Figure VII-4. Microtiter plate with results of VET-RPLA test. Sample 1 is negative; samples 2, 3, 4, 5, and 6 are positive.

assay detection level, no such lattice structure can be formed; therefore, upon settling a tight button will be observed.

2) Results in the column of wells containing control latex should be negative. The last well in all columns should be negative. If positive patterns are observed in some of these wells, the reaction should be regarded as invalid.
3) When excess CT is present, a prozone effect may be observed; i.e., a negative pattern is obtained in wells containing test sample and sensitized latex. However, as a result of the doubling dilutions, the concentration of CT in each well along the column is progressively reduced, negating the prozone effect due to excess amounts of CT. A positive pattern of agglutination will be seen after negative patterns in the first few wells of the column. With such results, the test sample should be classified as positive.
4) The sensitivity of this test kit in detecting CT is 1 to 2 ng/ml. Enterotoxin present at concentrations lower than this will, therefore, give negative results. The method is slightly less sensitive than the G_{M1}-ELISA, and, rarely, a toxigenic strain will give negative results.

H. PCR for CT Genes

The polymerase chain reaction is a technique that employs two short, specific DNA oligonucleotides (primers) and the enzyme DNA polymerase to synthesize multiple copies of DNA in the portion of the bacterial genome that is flanked by the two primers. In the PCR for cholera toxin described here, the specific primers detect only the gene encoding the A subunit of cholera toxin (*ctxA*; Figure VII-5). The amplified DNA from

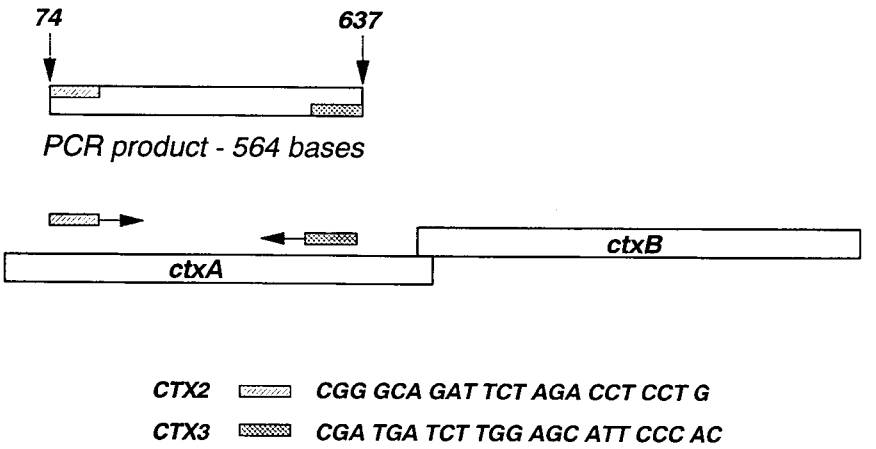

Figure VII-5. Location of the primers and the size of the amplified DNA fragment in the PCR assay for detection of *ctxA*.

ctxA is detected as a 564-bp band in an agarose gel. The PCR amplicon can be further characterized by restriction digests or hybridization with a specific internal probe to ensure that the amplicon band in the gel is from the *ctxA* gene. This PCR test is a colony confirmation test, designed for distinguishing toxigenic from nontoxigenic *V. cholerae* O1 strains taken from pure cultures. Others have described PCR assays that can detect toxigenic *V. cholerae* directly in stool or in food after enrichment in APW. Direct detection of *V. cholerae* by PCR in stool or food may be hampered by substances present in these types of samples which inhibit DNA polymerase.

Equipment and supplies

- Boiling water bath
- Thermocycler (Perkin-Elmer, Norwalk, Conn.; or M.J. Research Inc., Watertown, Mass.)
- Vortex
- Electrophoretic apparatus
- Power supply
- Camera and UV-transilluminator
- Micropipettes: 0.5-20 µl, 20-50 µl, and 100-1000 µl
- Sterile, disposable micropipette tips
- Microfuge tubes: 1.5 and 0.5 ml
- Gloves
- Dust/particle mask

Reagents

- Sterile water (make aliquots of 10-20 ml, open fresh aliquot for each assay)
- 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl, 0.1% [wt/vol] gelatin)
- 12.5x dNTP (2.5 mM each, dATP, dCTP, dGTP, dTTP in sterile distilled water)
- Primer CTX2: 5′ CGG GCA GAT TCT AGA CCT CCT G 3′; 100 µM in distilled water
- Primer CTX3: 5′ CGA TGA TCT TGG AGC ATT CCC AC 3′; 100 µM in distilled water
- AmpliTaq DNA polymerase (5 units/µl, Perkin-Elmer)
- Mineral oil
- Agarose (FMC BioProducts, Rockland, Maine)

- 10x Tris borate EDTA (TBE) buffer (108 g of Tris base, 55 g of boric acid, 40 ml of 0.5 M EDTA, and distilled water to 1,000 ml; adjust pH to 8.0)
- 10x gel loading buffer (0.5% sodium dodecyl sulfate, 10 mM EDTA, 50% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol)
- Ethidium bromide (stock solution is 10 mg/ml water)
- Quick spin columns (G-50, Boehringer Mannheim, Indianapolis, Ind.)
- Restriction endonucleases *Alu*I and *Rsa*I (Gibco BRL, Gaithersburg, Md.)
- 10x restriction buffer 1 (Gibco BRL)
- DNA molecular size marker: 1 kb ladder (Gibco BRL)
- TEMED (Sigma Chemical Co.)
- Ammonium persulfate (10% in water, made fresh daily, Gibco BRL)
- 30% acrylamide (29 parts acrylamide: 1 part bisacrylamide in water), store in dark at 4°C.
- Note: Acrylamide is neurotoxic; wear a face mask when preparing the stock solution.

Control strains

- *V. cholerae* ATCC 14035 (Classical, Ogawa, toxin-positive)
- *V. cholerae* ATCC 14033 (El Tor, Inaba, toxin-negative)

Performance of the test

SPECIAL NOTE: Only a very small amount of template DNA (DNA from which the PCR amplicon is generated) is required for PCR. Small amounts of contaminating DNA, especially the products of previous PCR assays, may result in false-positive results. It is important to use negative as well as positive controls in every experiment. Water in place of template DNA and a known negative strain are appropriate negative controls. Positive controls should be a known positive strain that is prepared along with the test strains and a known positive template preparation.

To reduce the possibility of contaminating the PCR test with the PCR products of previous reactions, the reactions should be prepared in one room and the amplification and electrophoresis should be performed in another room. The PCR product should never be taken into the PCR set-up room. A separate set of micropipettes, preferably positive displacement pipettes, should be reserved only for setting up PCR reactions.

1) To prepare a template DNA, suspend a 1-µl loopful of the control or the test strains in 0.5 ml of water to obtain a concentration of 10^5 to 10^6 organisms/ml. Boil the sample for 20 minutes to release DNA. One to 10 ng of DNA is sufficient; too much DNA can inhibit the am-

plification reaction. It is important to use water rather than PBS or any other phosphate-containing buffer because phosphate inhibits PCR. Heme-groups from blood agar plates can also inhibit PCR.

2) For each PCR test, a 50-µl reaction mix consists of 38.75 µl of sterile water, 5 µl of 10x PCR buffer, 4 µl of 12.5x dNTPs, 0.5 µl of each primer (1 µl total volume), 0.25 µl of AmpliTaq DNA polymerase, and 1 µl sample template. Prepare a "master mix" of all reagents except the sample DNA. This reduces pipetting errors and produces more consistent concentrations of reagents. Mix in one tube enough PCR buffer, dNTPs, *Taq* polymerase, primers, and water for all the tests being performed, and aliquot 49 µl of the master mix into each 0.5-ml microfuge tube. Then add 1 µl of the sample template.
3) Overlay the reaction mix with 1 drop of sterile mineral oil, and close the tubes. Program the thermocycler for a preincubation step at 95°C for 5 minutes, then 30 cycles of 1 minute at 95°C, 1 minute at 60°C, 1 minute at 72°C, and a final incubation at 72°C for 10 minutes. A final step of holding the tubes at 4°C can be added to refrigerate the samples until they are loaded on the gel. Place tubes in thermocycler and start.
4) Prepare a 0.8% agarose gel with TBE buffer. Mix 10 µl of the PCR mix and 1 to 2 µl of the 10x gel loading buffer, and load the wells. When removing the 10 µl of PCR reaction, make sure the pipette tip is beneath the oil layer. Use appropriate positive and negative controls with each set of PCR reactions, including a molecular size standard on each gel.
5) Run the gel until the bromphenol blue (purple color) has migrated about two-thirds of the way down the gel. Actual voltage and time will vary with the gel apparatus used (generally 2 to 3 hours at 60-80 V is sufficient). The 564-bp *ctxA* amplicon should migrate close to the bromphenol blue. Stain the gel with ethidium bromide (1 drop of stock solution in 500 ml of water) for 20 minutes, then destain in water for 10 to 20 minutes. [Note: ethidium bromide is mutagenic; wear gloves at all times.] Place the gel on the UV-transilluminator, and take photographs for documentation.

Interpretation of the results

The PCR test generates a 564-bp amplicon from the *ctxA* gene. This amplicon migrates just above the 0.5-kb band of the 1-kb DNA ladder size standard. The primers may be visible as a faint smear migrating just below the 200-bp size standard (Figure VII-6). The primers, but not the 564-bp amplicon band, will be visible for negative samples. Bands that are the wrong size should be considered negative. Faint bands that appear to be the correct size should be interpreted cautiously. When the results are in doubt, the amplified DNA can be verified as from the *ctxA* gene by digestion with restriction enzymes or hybridization with an internal probe

after Southern blotting. For routine applications, restriction analysis is simpler and faster than probing.

Verification of the PCR amplicon

1) Remove the mineral oil from the sample by placing the total volume of the PCR reaction on an angled parafilm strip. Mineral oil sticks to the parafilm as the aqueous drop slides downward. Purify the DNA fragment on a Quick Spin G-50 column. To prepare the column for use, centrifuge it at 600 x *g* for 5 minutes. Add the sample (10 to 50 µl) and repeat the centrifugation. The purified DNA will pass through the column in the same volume as was added to the column.
2) Transfer 15 µl of the PCR solution to a microcentrifuge tube; add 2 µl of water, 2 µl of restriction buffer 1 (BRL), and 1 µl of *Rsa*I or *Alu*I (or both). Incubate for 2 hours at 37°C.
3) Separate the restricted fragments by electrophoresis in an acrylamide gel. Acrylamide gels produce better resolution of small DNA fragments. For a minigel apparatus, prepare an 8% gel with 6.6 ml of 30% acrylamide (29:1 acrylamide:bisacrylamide in water), 15.6 ml of water, 2.5 ml of 10x TBE, 0.2 ml of 10% ammonium persulfate, and 20 µl of TEMED. Pour the gel and let it polymerize for 1 hour before loading the samples (10 µl of the PCR reaction and 1 µl of 10x loading buffer). Let the bromphenol blue migrate half way down the gel before staining DNA, as for an agarose gel. Restricting the 564-bp *ctxA* amplicon with *Rsa*I generates three fragments of 480, 70, and 14 bp; the 14-bp fragment may not be visible. *Alu*I produces two

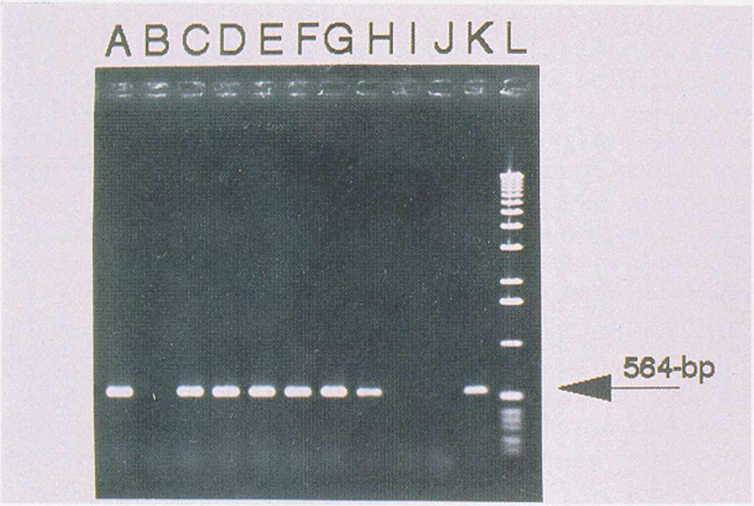

Figure VII-6. Agarose gel with results of a typical cholera toxin PCR test. Lanes A, C, D, E, F, positive test strains; lanes B, J, negative test strains; lanes G, K, positive controls; lanes H, I, negative control strains; lane L, I-kb DNA ladder.

fragments of 499 and 65 bp. Combination of the two enzymes generates four fragments: 415, 70, 65, and 10 bp.

I. DNA Probes for CT Genes

In colony blots, isolated colonies to be tested for cholera toxin genes are inoculated in a grid-fashion on a nonselective agar plate and incubated until the patches of growth reach the desired size. The patches are then transferred to a nylon filter, where they are treated by a series of steps that lyse the cells and fix the denatured or single-stranded DNA to the filter. The filter is then hybridized with a specific DNA probe that corresponds to the *ctx* gene. Although cloned DNA and oligonucleotides have been tested, a PCR-generated amplicon that contains digoxigenin-labeled bases has proved to be sensitive, stable, and safe.

1. Generation of digoxigenin-labeled PCR amplicons

The procedure for generating digoxigenin-labeled PCR probes is similar to that for detecting *ctx* genes with PCR (Section H of this chapter), except that a well-characterized CT-positive strain of *V. cholerae* O1 is used to produce the template DNA and a second amplification is used to incorporate the digoxigenin-labeled base.

Equipment and supplies

See Section VII-H (PCR for CT genes)

Reagents

- See Section VII-H (PCR for CT genes)
- 10x "–digoxigenin" dNTP mix (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP)
- Digoxigenin-11-dUTP (Boehringer Mannheim; concentration is 1 mM)

Procedure

1) Generate the PCR amplicon that will be used as the template in a labeling PCR by following the procedure described in Section VII-H using a known CT-positive strain (ATCC 14035 or another well-characterized, CT-positive strain) as the source of template DNA. Incorporation of digoxigenin-labeled dUTP into a DNA molecule is less efficient than incorporation of standard dNTPs; therefore, the PCR amplicon is used as template DNA rather than total genomic DNA in the labeling PCR to increase the amount of labeled DNA (probe) produced.
2) Run 5 to 10 µl of the PCR mix on a 0.8% agarose gel to confirm production of the appropriate size and concentration of the DNA amplicon. Stain and photograph for documentation.

3) Make a dilution of the PCR mix so that about 50 ng of the PCR fragment is used as the template in the labeling PCR (usually, 1 to 3 µl of a 1:10 dilution).
4) Set up the labeling PCR in a 0.5-ml microcentrifuge tube: 1 to 3 µl of the diluted PCR-generated template DNA, 1 µl of each primer (2 µl total volume), 10 µl of 10x PCR buffer, 10 µl of 10x "–digoxigenin" dNTP mix, 7 µl of digoxigenin-11-dUTP (1mM), 0.5 µl of *Taq* polymerase, 67.5 to 69.5 µl of sterile distilled water. Overlay the mixture with 2 drops of mineral oil.
5) Program the thermocycler for 40 cycles of 30 seconds at 95°C, 1.5 minutes at 60°C, 3 minutes at 72°C; then a 10-minute incubation at 72°C; hold at 4°C. Place tubes in thermocycler and start.
6) Remove unincorporated dNTPs and primers by purifying the DNA over a Quick Spin G-50 column.
7) Run 2 to 3 µl of the PCR mix on a 0.8% agarose gel. Stain and photograph. The digoxigenin-labeled amplicon will migrate more slowly (i.e., at a higher apparent molecular weight) than the nonlabeled amplicon because of the presence of digoxigenin in the molecule.

2. Colony hybridization for cholera toxin genes

Equipment and supplies

- 8 cm diameter circular nylon filters (Micron Separations, Inc., Westboro, Mass.)
- Whatman 3MM chromatography paper
- Drying oven
- Platform shaker
- Plastic, heat-sealable bags
- Heat-sealing apparatus
- Petri dishes
- Luria broth (LB) plates or other rich medium (do not use MacConkey plates because they seem to cause more background staining on the blots)

Reagents

- Digoxigenin-labeled probe (amplicon prepared as in section I.1.)
- 10 N NaOH
- 1 M Tris, pH 8.0
- 5 M NaCl
- 20% SDS
- 20x SSC (175.3 g NaCl, 88.2 g sodium citrate in 1 liter water, pH 7.0)

- Prehybridization solution: 5x SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 0.02% SDS (can be prepared in large quantities in advance, aliquoted, and stored at -20°C) [Note: Prehybridization solution is used for both prehybridizations and hybridizations.]
- Buffer A (100 mM Tris, pH 7.5, 150 mM NaCl)
- Buffer C (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$)
- Nonfat dry milk
- Anti-digoxigenin Fab fragment/alkaline phosphatase conjugate (Boehringer Mannheim)
- Nitroblue tetrazolium (NBT) (75 mg/ml in water)
- 5-bromo-4-chloro-3-indolylphosphate toluidinum (BCIP) (175 g/ml in water)

Preparation of colony blots

1) Transfer isolated colonies of each test strain to a dry LB plate using a grid to organize the colonies. Incubate the plate for several hours or overnight at 37°C until all the colonies are clearly visible on the plate.
2) Mark an 8-cm circular nylon filter (50 cm^2 total area) with an arrow to orient the position of the colonies and a number or date to identify the filter. Place the filter (labeled side down) over the inoculated LB plate with the arrow at the top of the plate. Remove any trapped air bubbles. Incubate the LB plate for 0.5 to 2 hours at 37°C. [Note: Nitrocellulose filters can also be used, but they tear more easily than nylon filters. Glass filters, such as Whatman 541, cannot be used in this assay because digoxigenin sticks nonspecifically to these filters.]
3) Saturate two sheets of 3MM chromatography paper with 0.5 M NaOH in a glass pan. Lift the nylon filter off the plate and place on the 3MM paper, colony side up. Visible patches of each colony should be stuck to the filter. Allow the cells to lyse for 15 minutes.
4) Saturate two sheets of 3MM chromatography paper with 1.0 M Tris, pH 8.0, in a glass pan. Transfer the filter from the paper saturated with NaOH to the paper saturated with Tris, again colony side up. Allow neutralization to occur for 10 minutes.
5) Saturate two sheets of 3MM chromatography paper with 0.7 M Tris 1.5 M NaCl, pH 8.0, in a glass pan. Transfer the filter from the paper saturated with 1.0 M Tris to the paper saturated with Tris/NaCl colony side up. Allow neutralization to occur for 10 minutes.
6) Rinse the filter briefly in 2x SSC. Blot the filter on dry 3MM chromatography paper and air dry at room temperature or 37°C. Bake the filter at 80°C in an oven for 0.5 to 2 hours. The filter is now ready for

hybridization but it can be stored in in an airtight container at room temperature until needed.

Hybridization of colony blots

1) Place the filter in a plastic, heat-sealable bag. Add 10 ml (0.2 ml/cm^2) of prehybridization solution. Remove air bubbles by rolling a pipette across the bag and squeezing the bubbles to the top. Do not squeeze the fluid out of the bag. Seal the bag with a heat sealing apparatus. Incubate the filter in the bag at 65°C for 1 hour in a shaking water bath.
2) Prepare the probe 15 to 20 minutes before the end of the prehybridization time (see section I.1. for instructions for probe preparation). Dilute the probe in a total volume of 100 µl in a microfuge tube; use 1 to 5 µl of probe, depending on the concentration. [Note: The optimal concentration of probe may need to be determined empirically by testing several concentrations on replicate filters.] Denature the probe by incubating in a boiling water bath for 10 minutes. Quickly cool the probe by placing it on ice for a few minutes. Centrifuge the probe in a microfuge for a few seconds, and hold on ice until needed.
3) Remove the filter from the water bath. Cut one corner of the bag and remove all the prehybridization solution from the bag. Add 2.5 ml (0.05 ml/cm^2) of prehybridization solution and the probe to the bag. Remove the air bubbles and seal the bag so that it conforms to the shape of the filter as closely as possible to maximize contact between the filter and the probe.
4) Hybridize the filter at 65°C overnight in a shaking water bath.
5) Prepare about 500 ml of wash solution (1x SSC, 0.1% SDS), and preheat to 65°C. Remove the filter from the plastic bag and place in a glass pan or dish. Add 100 ml of wash solution to the glass pan and rinse the filter briefly. Pour off the used wash solution.
6) Add 200 ml of wash solution to the glass pan and wash the filter at 65°C in a shaking water bath for 15 minutes. Pour off the used wash solution. Add the remaining wash solution and incubate again at 65°C for 15 minutes. Air dry the filter and hold for later development, or proceed directly to the developing steps.

Detection of digoxigenin-labeled probes

1) Rinse the filter with 40 ml of buffer A in a petri dish on a platform shaker for 1 minute.
2) Remove the filter from buffer A, allowing excess solution to drip off for a few seconds. Place the filter in another petri dish containing 40 ml of buffer A + 5% nonfat dry milk (blocking solution). Incubate with gentle shaking for 1 hour.

3) Pour off the blocking solution and replace with the anti-digoxigenin/alkaline phosphatase conjugate diluted 1:5000 in 10 ml of buffer A + 5% nonfat dry milk. Incubate for 30 minutes with gentle shaking.
4) Transfer the filter to a petri dish containing 40 ml of buffer A. Incubate with gentle shaking for 15 minutes. Repeat the wash step once.
5) Rinse the filter in a petri dish containing 40 ml of buffer C on a shaker tray for 2 minutes.
6) Remove the filter from buffer C, allowing excess solution to drip off for a few seconds. Place the filter in a petri dish containing 10 ml of buffer C with 45 µl of NBT (75 mg/mL) and 35 µl of BCIP (175 µg/ml). Seal the petri dish with parafilm. Place the petri dish in a dark place and check periodically. Results may be observed within a few hours, although overnight exposure may be required for complete development.

Interpretation of results

ctxA-positive isolates will appear as dark purple/brown patches on the nylon filters (Figure VII-7). Negative controls and negative isolates may be visible as very faintly stained patches.

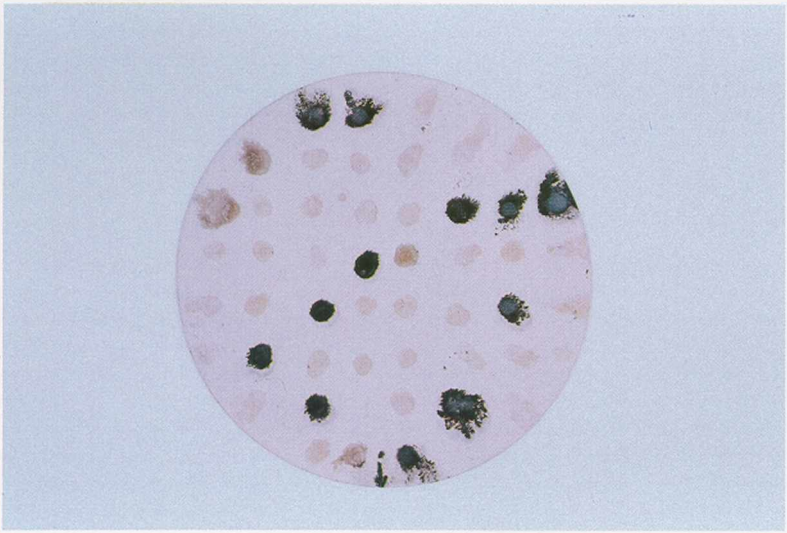

Figure VII-7. Results of hybridization with a digoxigenin-labeled probe for *ctxA*; dark purple color indicates a positive reaction.

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VIII. Detection of Patient Antibodies to *Vibrio cholerae* O1 and Cholera Toxin

Isolation of *Vibrio cholerae* O1 from a patient's stool is the definitive proof of infection with the organism. However, in some circumstances, such as when a stool specimen is not obtained while the patient is symptomatic or is obtained after the person has stopped excreting the organism, either because of antibiotic treatment or the passage of time, isolation of the organism is not possible. Likewise, isolation may not be possible if the specimen is mishandled and the vibrios die, or if there is no laboratory support for bacteriologic studies. In these situations, the presence of, or change in, serum antibodies can often determine if a person has been infected with *V. cholerae* O1.

Because most *V. cholerae* O1 infections are asymptomatic or mildly to moderately symptomatic, a survey of clinical illness is not a good way to determine what proportion of a population has recently been infected with *V. cholerae*. Serum antibodies can provide the answer. Antibody assays can be particularly valuable for 1) identifying for case-control studies control subjects who have not had recent *V. cholerae* O1 infections, 2) determining, in areas without confirmed cholera, if cases of suspected cholera that escaped detection by culture were indeed infections with *V. cholerae* O1, 3) determining the proportion of persons in newly affected areas who have had *V. cholerae* O1 infections, and 4) using stored serum collections to determine if cholera existed undetected in an area in previous years.

The two most commonly performed assays for detection of antibodies in *V. cholerae* O1 infections are vibriocidal antibody assays (antibodies against somatic O antigens of *V. cholerae*) and assays for antitoxic antibodies (antibodies against cholera toxin). A summary of these methods is presented in Table VIII-1.

A. Vibriocidal Test

The vibriocidal test is a reliable and well-documented method for determining the presence of bactericidal antibodies directed against O antigens of *V. cholerae* O1. In the test, dilutions of patient serum are mixed with a standardized inoculum of *V. cholerae* O1 (both Ogawa and Inaba should be used) and excess guinea pig complement. During the initial incubation period, bacterial cells are killed if vibriocidal antibodies are present. Culture medium is added, and a second incubation period follows to detect surviving bacterial cells. If turbidity develops in the culture medium, it indicates the absence of vibriocidal antibodies or at least an amount of antibodies beneath the threshold of detectibility with this method.

Several crucial elements of the vibriocidal test can cause the assay to fail. Carefully performed quality control and strict adherence to the

Table VIII-1. Vibriocidal and anti-CT Methods

Test characteristic	Vibriocidal test	Anti-CT ELISA
Specific reagents required	Guinea pig complement, *V. cholerae* Inaba and Ogawa serotype cultures	Cholera toxin, enzyme-conjugated antibody to human IgG, enzyme substrate
Controls required	Positive and negative human control antisera	Positive and negative human control antisera
Type of assay	In vitro killing of *V. cholerae* in the presence of complement	Binding of antibody to antigen on solid phase detected by enzyme-conjugated antiglobulin antibody
Antibody measured	Antibacterial antibodies	Anti-cholera toxin antibodies
Peak antibody response	10 days after onset of illness	21-28 days after onset
Duration of antibodies as measured by test	Start to decline after 1 mo.; disappear in 12 mos.	May persist for as long as 2 years
Advantages	Sensitive; requires only 1 commercial reagent; doesn't require expensive equipment	Anti-CT antibody is not stimulated by killed-cell parenteral cholera vaccine
Disadvantages	Vibriocidal antibody affected by current cholera vaccines; standardization of inoculum crucial for accuracy of test; live cultures used	Not as sensitive as the vibriocidal test; requires several commercial reagents; antibody to *E. coli* LT may affect accuracy of the test

Note: LT = *Eschericia coli* heat-labile enterotoxin.

protocol are essential for reproducible results. Problems with the vibriocidal test often occur with the following:

1) source, storage, or dilution of complement
2) *V. cholerae* O1 standard strains
3) preparation of the antigen suspension
4) incubation steps during the performance of the test.

1. Preparation and quality control of reagents for the vibriocidal test

Complement

Freeze-dried guinea pig complement is available from commercial sources. Some laboratories may find it impractical or impossible to purchase freeze-dried guinea pig complement and may wish to prepare their own supply from local animal sources.

Preparation of fresh, pooled complement

Obtain 10 ml of blood from individual healthy guinea pigs by cardiac puncture. Allow the blood to clot (1 hour at room temperature), centrifuge, and promptly separate the serum from each animal into an individually labeled tube. At this point, the sera may be frozen individually for later testing before pooling or held continuously in an ice bath while testing is completed within 24 hours. (See below.)

Testing of individual guinea pig sera for intrinsic vibriocidal activity

When fresh, pooled guinea pig serum is used as a source of complement in the vibriocidal antibody test, it should not have intrinsic vibriocidal activity that could interfere with the test by killing the inoculum. Occasional guinea pigs (perhaps one in 20) from some animal colonies have low levels of antibodies reacting with *V. cholerae*, presumably because they have become colonized or infected with an antigenically cross-reacting species of bacteria. Commercial guinea pig complement generally represents a very large pool of serum from many guinea pigs, and any antibodies present usually become diluted in the pool below an effective level of interference in the test unless the prevalence of naturally occurring antibodies against *V. cholerae* is high in the original guinea pig colony.

Serum is screened for intrinsic complement-dependent vibriocidal activity by comparing the killing power of unheated serum with that of the same serum heated at 56°C for 30 minutes to destroy complement activity. Naturally occurring antibody against *V. cholerae* will not kill the organism if complement activity has been inactivated.

The following pretesting procedure may be helpful in eliminating unsuitable serum when preparing pooled guinea pig complement:

1) Take 0.1 ml of each guinea pig serum and dilute 1:5 by adding it to 0.4 ml saline in a small labeled test tube. Mix well and pipette 0.25 ml into a separate labeled tube (marked "heated"). Keep the original tube in an ice bath while the second tube is heated in a water bath at 56°C for 30 minutes.
2) For each serum sample to be tested, add 15 µl of unheated diluted serum to a microtiter plate well and add 15 µl of the heated sample of the same serum to an adjacent well.
3) Add 15 µl of *V. cholerae* suspension (standardized and diluted 1:10), as specified in Section, "Preparation and standardization of the antigen suspension."
4) Add 30 µl of sterile saline to each well and incubate at 37°C for 1 hour.
5) Add 150 µl of warm heart infusion broth to each well and incubate for 1 hour and 45 minutes.
6) For each serum tested, compare the turbidity in adjacent wells containing heated and unheated samples.
7) Identify and discard any guinea pig serum that shows significantly more turbidity in the "heated" versus the "unheated" wells. Such sera should not be used in the vibriocidal test but may be used for other complement-dependent reactions.)
8) After eliminating individual sera that appear to have native vibriocidal activity, pool the remaining sera, mix well, and freeze.

Note: If desired, the test can also be performed in 13 x 100-mm or 12 x 75-mm test tubes by adjusting quantities of inoculum, saline, and broth. This would save a microtiter plate if only a small number of guinea pig sera are being tested.

Titration of complement

Unlike the complement fixation test, it is not necessary to titrate the complement for the vibriocidal assay because an excess of complement is used.

Storage of complement

Complement should be distributed and frozen in convenient aliquots in tightly stoppered vials. One milliliter of guinea pig complement will provide sufficient diluted complement for four microtiter plates. Commercial complement should also be aliquoted and frozen immediately after rehydration. [Warning: Do not use the commercial diluent provided with the complement to rehydrate because it may contain a preservative.] Complement can be stored at –70°C for at least 1 year and at –20°C for at

least 3 months. When thawed, the complement must be used or discarded, and should not be refrozen.

Standard strains

The standard control strains used at CDC are VC 12 (*V. cholerae* O1 Ogawa) and VC 13 (*V. cholerae* O1 Inaba). Other well-characterized Inaba and Ogawa strains may be used, but the use of VC 12 and VC 13 would allow standardization of results from many laboratories.

The standard strains should be stored frozen, preferably at –70°C, and streaked out on heart infusion agar (HIA) or another nonselective medium to prepare working slants as needed. Working slants should be stored for no more than 2 weeks to ensure that the strains remain smooth and viable for the assay.

Preparation and standardization of the antigen suspension

Two antigen suspensions need to be prepared, one from VC 12 and one from VC 13. Suspend fresh growth from each strain in 2 to 3 ml of 0.85% saline and then carefully adjust the turbidity to a density of approximately 1.5×10^9 CFU/ml. This is the most critical element of the vibriocidal assay; an antigen suspension that is either too turbid or too dilute can affect the titers obtained from the assay.

McFarland method

Before using this method, a McFarland 4 standard (see Chapter XI, "Preparation of Media and Reagents," for the formula for preparation of the McFarland 4 turbidity standard) should be prepared, test antigen suspensions (for both VC 12 and VC 13) should be made and adjusted to the same turbidity as the McFarland 4 standard, and plate counts should be done to ensure that the turbidity of the McFarland 4 is correct and is the density of 1.5×10^9 CFU/ml.

Agitate a McFarland 4 turbidity standard on a vortex mixer immediately before use. Visually compare the McFarland standard with the antigen suspension prepared as described above, and dilute if necessary with sterile saline. (For proper turbidity adjustment, use a white background and a contrasting black line in combination with an adequate light source.)

Spectrophotometer method

Before using this method, the optical density (OD) corresponding to 1.5×10^9 CFU/ml must be established by performing plate counts and plotting the OD (wavelength 550 nm) versus plate count to obtain a standard curve. With a 1-cm cuvette and a 1-cm light path, the OD at this concentration should be 0.9 to 0.95. Once this OD is known, the standard curve does not need to be redrawn as long as the same spectrophotometer, cuvettes, and diluent are used. After preparing suspensions from slants

(described above), measure OD at 550 nm of each suspension and adjust with sterile saline until the proper OD is achieved.

2. Vibriocidal test procedure

If desired, the vibriocidal test can also be performed in tubes by adjusting quantities of inoculum, saline, and broth.

Inoculation of test strains

1) The afternoon before the test, streak out VC 12 and VC 13 (or other well-characterized Ogawa and Inaba control strains) from working stock cultures to fresh HIA plates (or other nonselective medium) and incubate at 35° to 37°C for 16 to 18 hours. Place four uninoculated slants in a 35°–37°C incubator to prewarm. Inoculate the prewarmed slants with the overnight cultures of VC 12 and VC 13 (two slants per strain). Spread heavily and evenly over surface of slants. Incubate 4 hours at 35° to 37°C.

Preparation of serum dilutions

2) Always process a positive serum of known titer and a negative serum with each day's run. Sterile saline is used as diluent. Prepare an initial 1:5 dilution of each test and control serum by placing 25 µl of serum in the first well in the row of a 96-well round-bottom microtiter plate and adding 100 µl saline.
3) Place 25 µl of saline in each well of the remaining rows.
4) Transfer 25 µl from the first well (1:5 serum dilution) to the second well (first test well). Make serial 25-µl twofold dilutions through well #12 using a multichannel pipette. The first test dilution (well #2) will be 1:10 (1:20 after adding antigen).

Standardization of antigen suspension

5) After slants have been incubated for 4 hours, wash growth from slants with 2 to 3 ml sterile saline (0.85%). Adjust the turbidity of these suspensions to a density of approximately 1.5×10^9 CFU/ml by one of the previously described methods. The density-adjusted suspensions are diluted 1:10 in sterile saline (giving a density of approximately 1.5×10^8 CFU/ml) to provide the antigens for use.

Preparation of complement–bacteria mixture

6) Dilute freshly thawed complement 1:5 in sterile distilled water. [Warning: If using commercial complement, do not use the diluent provided with it because it may contain a preservative.]

7) Mix equal amounts of the 1:5 diluted complement and the 1:10 diluted standardized bacterial antigen suspension (see section, "Standardization of antigen suspension," above). The mixture should be kept on ice and used within 30 minutes. About 2.5 ml of bacteria–complement mixture is needed for each plate. The final bacterial concentration in the mixture is approximately 7.5×10^7 bacteria/ml.

Addition of complement–bacteria mixture

8) Add 25 µl of complement–bacteria mixture to serum dilutions prepared earlier in the microtiter plate. The complement–bacteria mixture should be added to all serum dilutions except those in first column, which are used to detect bacterial contamination of the serum.

9) Mix by shaking, and seal microtiter plates with plate sealers.

10) Place in 35° to 37°C water bath for 1 hour (If a water bath is not available, a 35° to 37°C incubator may be used; however, the incubation time may need to be extended.) Prewarm the heart infusion broth for the next step.

Addition of heart infusion broth

11) Remove from water bath or incubator and add 150 µl of warm heart infusion broth (pH 7.4) to each well, including the wells in column 1, which will be used as controls to detect serum contamination. (If the serum is contaminated, the test is invalidated.) Return the plate to the 35° to 37°C water bath and incubate for 1 hour and 45 minutes.

12) Remove plates from the water bath and read test. If growth in negative serum control wells is light, re-incubate for 15 to 20 minutes before reading the test.

Note: Adhere strictly to incubation times, especially for the step in which the complement–antigen mixture is added to the dilutions of antisera.

Reading of microtiter vibriocidal test

13) Read the test by looking for visible growth or turbidity in the wells after adding broth and incubating. The absence or inhibition of growth, as evidenced by lack of turbidity, is a positive reaction (Figure VIII-1). The titer is obtained by determining the serum dilution that inhibits approximately 50% of the growth of *V. cholerae.*

Note: The test titer of the positive control serum should be within one dilution of its expected titer. If the well in the first column, which contains only serum plus broth, shows growth, the serum is contaminated. If the entire microtiter plate shows growth, the broth is contaminated.

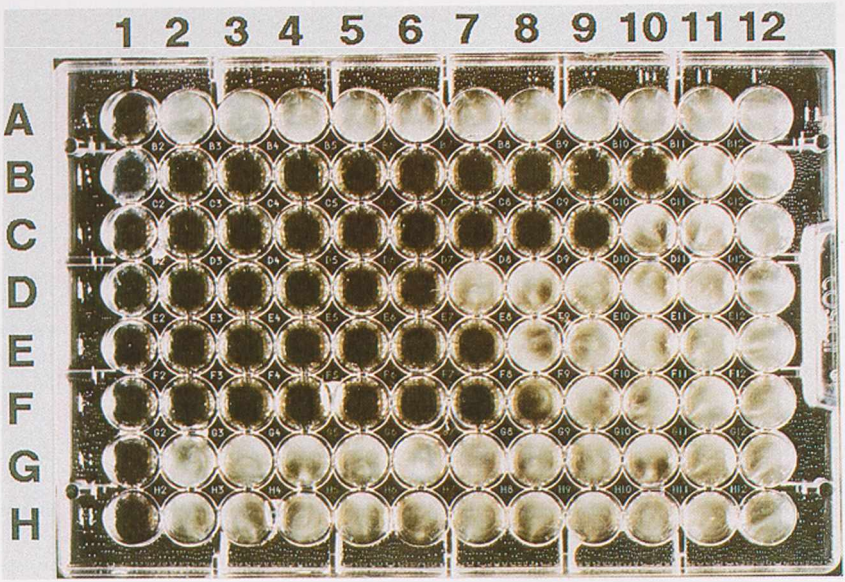

Figure VIII-1.The absence or inhibition of growth indicates that the *V. cholerae* O1 strain has been killed by the action of specific antibodies in the patient's serum. Row A contains the negative control serum and row B, the positive control serum. The sera in rows A, G, and H are negative for vibriocidal antibodies since growth has occurred in all wells but column 1, which is the control for serum contamination. The titers of the other rows are as follows: B, 5120; C, 2560; D, 320; E, 640; F, 1280 (in well #8, the turbidity is ≤50% of the negative control well, so it is read as positive).

B. ELISA for Cholera Antitoxin

Cholera toxin (CT) is antigenic, and its immunobiologic properties have been well characterized. Persons who have been infected with CT-producing *V. cholerae* usually develop a marked antibody response to the toxin. This immune response is the basis of a serologic examination to determine the antibody status of an individual to CT, and thus establishes a reliable indicator of recent infection with toxigenic *V. cholerae*. Antibodies to CT can be determined in animal and tissue culture assays, which determine if the CT activity has been neutralized by the patient's serum; however an anti-CT enzyme-linked immunosorbent assay (ELISA) is commonly used by laboratories that are unable to perform in vivo assays or tissue culture. Interpretation of anti-CT antibody assays is complicated by the fact that CT is antigenically similar to the heat-labile enterotoxin (LT) produced by *Escherichia coli,* and cross-reactions could occur in persons who have had recent infections with LT-producing *E. coli*.

The ELISA for antibodies to CT is based on the binding of those antibodies in patient serum to immobilized CT. By screening paired serum samples collected early in the illness (acute-phase specimen) and during the convalescent period (convalescent-phase specimen), pre- and post-

illness antibody levels against CT can be measured and compared. In the microtiter test procedure, microtiter wells are coated with CT and carefully washed, after which the patient's serum is added to the wells. If anti-CT antibodies are present, they will bind to the CT and be immobilized in the microtiter well. After further washing, goat anti-human IgG that has been labeled with an enzyme (such as alkaline phosphatase or horseradish peroxidase) active on a chromogenic substrate is added to the well. The goat anti-human IgG binds to the human immunoglobulin. When the wells are washed again to remove all unbound materials, only the "sandwich" of CT + patient anti-CT + enzyme-labeled goat anti-human IgG remains. The chromogenic substrate is then added, and the amount of bound enzyme is quantitated by measuring the intensity of the color reaction. The intensity of the color reaction is proportional to the quantity of anti-CT in the patient's serum.

Anti-CT ELISA procedure

1) Dilute cholera toxin (Calbiochem Corp., La Jolla, Calif.) to 1 μg/ml in carbonate buffer (0.05 M, pH 9.6). Place 100 μl of the solution in each of the inner 60 wells of a flat-bottom or round-bottom, 96-well microtiter plate.
2) Place covered plates in a moist chamber and refrigerate overnight (15 to 20 hours).
3) Wash each plate four times with 0.01 M phosphate-buffered saline, pH 7.2, plus 1% Tween (PBS–T). Leave last wash in plates and store in a moist chamber at 4°C for later use. Plates should not be stored for more than a few days.
4) Dump last wash from plate and block all wells with 150 μl of PBS–T containing 0.5% nonfat dry milk and 1% fetal bovine serum (PBS–T–M–FBS). Incubate at room temperature for at least 1 hour. Dump fluid, but do not wash. The plate has now been "sensitized" for further use.
5) In small sterile tubes, make 1:200 dilutions in PBS–T–M–FBS (5 μl serum in 1 ml buffer) of each serum to be tested. Include one positive and three negative control sera with each run.

   If you wish to know only whether a given serum is positive for antitoxic antibodies, no further dilutions need to be made. Dispense 100 μl of each serum diluted 1:200 to the corresponding wells in the sensitized microtiter plate (prepared in steps 1 through 4).

   If you wish to determine an antibody titer, dilute sera in a separate, unsensitized microtiter plate as follows: place 200 μl of the 1:200 diluted serum in the first column of inner wells; place 100 μl of PBS–T–M–FBS in each of the remaining wells; make six serial 100-μl twofold dilutions using a multichannel pipette. Dispense 100 μl of each serum dilution to the appropriate wells in the sensitized plate

(prepared in steps 1 through 4). Note: The negative control sera are not diluted.

6) Incubate at 35° to 37°C for 1 hour.

7) Dump fluid from plates and wash four times with PBS–T containing 1% fetal bovine serum (PBS–T–FBS).

8) Dilute alkaline-phosphatase-labeled anti-human conjugate (1:2000; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) in PBS–T–FBS, and place 100 µl in each well. [Note: Since the conjugate may vary from lot to lot, the concentration used should be determined by titration before use.] Incubate for 1 hour at 35° to 37°C. Dump fluid and wash 4 times with PBS–T–FBS.

9) Prepare the alkaline phosphatase substrate solution (*p*-nitrophenyl phosphate [Sigma Chemical Co., St. Louis, Mo.] in diethanolamine buffer; see Chapter XI, "Preparation of Media and Reagents," for preparation instructions). Place 100 µl of the enzyme substrate solution in each well and incubate at room temperature for 30 minutes.

10) Stop reaction by adding 50 µl 3 M NaOH to each well. Mix well. Read optical density at 405 nm on an ELISA reader. Record results.

If titers are not determined (i.e., a single 1:200 dilution is used), an OD reading of 0.30 or greater may be considered a significant or "positive" reading (see Section C below, "Interpretation of Results from Serologic Tests").

An optical density greater than 0.1 (or OD 2x greater than the mean of the three negative control sera) is considered positive for the presence of cholera antitoxin at that dilution. The serum titer is the reciprocal of the last dilution giving a positive OD reading. An antitoxin titer of 400 is considered positive, whereas 200 is considered borderline (see Section C below).

C. Interpretation of Results from Serologic Tests

When possible, paired sera for vibriocidal antibodies should be collected from each person, with the acute-phase serum obtained as early as possible (ideally within 0 to 3 days after onset) and the convalescent-phase serum obtained 10 to 21 days after onset. A fourfold (two dilution) or greater rise in vibriocidal antibody titer to at least 320 is diagnostic of *V. cholerae* O1 infection if the patient was not recently given cholera vaccine. Lower titers may not be reproducible. Vibriocidal antibodies develop in at least 90% to 95% of persons with culture-confirmed *V. cholerae* O1 infection. Vibriocidal titers rise rapidly after onset of illness or infection. The increase in titers is similar for all infected persons, regardless of the presence or severity of symptoms. Titers in human volunteers usually peak within 10 days after ingestion of the organisms, begin to decline within 1 month, fall markedly by 6 to 12 months, and are almost back to baseline levels after 1 year.

At no level is a single vibriocidal titer diagnostic of cholera in all situations. In the United States, where cases of cholera are exceedingly rare, one study showed that anti-Ogawa titers of >640 occur in only 0.9% of healthy persons. In a volunteer study, at 1 month after infection, 79% of persons had titers of ≥1,280, 89% had titers of ≥640, 92% had titers of ≥320, 97% had titers of ≥160, and 99% had titers of ≥20. A person with a titer of <20 is highly unlikely to have had a *V. cholerae* O1 infection during the previous 10 to 30 days, whereas a person with a titer of ≥1280 is likely to have been infected with the organism and is considered to be "positive" for most purposes. Persons with intermediate titers may or may not have been infected, and the level that should be considered "positive" depends on the degree of certainty desired. For example, while vibriocidal titers of 160 and 320 are usually considered "negative," persons with these titers are usually not accepted as "negative" controls in cholera case-control studies. A vibriocidal titer of 640 is borderline (questionably) positive. A titer of 640 may represent past *V. cholerae* O1 infection or antibodies due to infections with organisms capable of inducing cross-reacting antibodies (*Yersinia enterocolitica* serotype O9, *Brucella* spp., *Citrobacter* spp., and other cross-reacting members of the *Enterobacteriaceae*.) Borderline results should be repeated and confirmed because a variation in titer by one tube or dilution may normally be encountered between runs with the same specimen. A decline in titer from the acute-phase serum to the convalescent-phase serum may also indicate infection.

Interpretation of antitoxic antibody assays is complicated by the fact that cholera toxin is similar to LT produced by *E. coli*. This is rarely a problem in countries like the United States where enterotoxigenic *E. coli* infections are unusual. However, in most countries where cholera is common, infections with enterotoxigenic *E. coli* are also common, and antitoxic antibody assays are less useful than they might be. It is possible to distinguish between antibodies produced against the two different toxins by testing sera against both CT and LT, but these procedures are beyond the scope of this manual and should be sought elsewhere.

Antitoxic antibodies have been detected by the anti-CT ELISA in 88% of volunteers excreting *V. cholerae* O1. The antibodies can remain elevated for over 2 years. Antitoxic IgG antibodies peak at 21 to 28 days after onset of illness. The acute-phase serum sample for antitoxic antibody testing should be collected as early as possible (ideally within 0 to 3 days after onset), and the convalescent-phase serum should be obtained as close to 21–28 days after onset as possible. A net OD increase of 0.2 or greater between the acute- and convalescent-phase sera (at a 1:200 dilution) or a fourfold rise in titer represents a significant antitoxin response. A single specimen is less useful, but a net OD of ≥0.30 (at a single dilution of 1:200) has been used to indicate a positive specimen. A serum titer of 400 may also be considered positive. A serum titer of 200 is borderline (questionably) positive.

To determine if a person has had a recent *V. cholerae* O1 infection, testing a single serum specimen by using both the vibriocidal and antitoxin assays can provide a more secure diagnosis than testing with either assay alone. The likelihood of a rise in titer to two independent antigens of *V. cholerae* occurring by chance in a noninfected person is remote.

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IX. Molecular Subtyping of *Vibrio cholerae* O1

Molecular biological techniques have found several applications in diagnostic and public health microbiology. These techniques are generally more rapid and more specific than traditional techniques, and often offer better discrimination between strains. Molecular techniques have been important in the subtyping of *Vibrio cholerae* isolates and appear most useful in reference laboratories where specialized equipment and reagents are available. These techniques have the distinct advantage of being generic, or applicable to a wide range of bacterial pathogens. Although most methods require qualitative measures and interpretation of data, this should change with the increased use of bioimaging equipment and computerized data analysis. Because genotypic assays have become more important in clinical microbiology, efforts are under way to standardize the methods.

The routine isolation and identification of *V. cholerae* O1 is relatively straightforward. However, genotypic techniques are useful for characterizing unique strains since phenotypic characteristics alone are not usually sufficient to discriminate among strains of *V. cholerae* O1. Since strains from the Latin American epidemic could not be subtyped using phenotypic characteristics, they were defined by multilocus enzyme analysis, pulsed-field gel electrophoresis, ribotype, and other molecular subtyping methods. The relationships of Latin American isolates to those from the U.S. Gulf Coast and the rest of the world have also been studied.

A. Plasmid Profiles

Plasmids are extrachromosomal DNA elements that mediate various phenotypic traits and may mediate conjugation between bacterial cells. Some plasmids are autotransmissible and may stimulate the transfer of other plasmids as well. These genetic properties often result in strains that possess a complex collection of plasmids; the number and size of these plasmids can be determined by isolation of plasmid DNA and agarose gel electrophoresis. This plasmid profile may be unique to specific strains of bacteria and has been useful in identifying the epidemic strain in outbreaks of diarrheal disease.

Plasmid profiles were particularly useful in characterizing *V. cholerae* isolates of the classical biotype, which usually have plasmids of 21 and 3 megadaltons. However, El Tor biotype strains of *V. cholerae* O1 seldom have plasmids not associated with acquired antibiotic resistance.

B. Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphisms (RFLPs) are differences in the size of specific DNA restriction fragment(s) within different strains. A restriction enzyme that recognizes specific target sequences (usually 4 to 8 base pairs) within the DNA molecule cuts or digests total genomic DNA

into discrete lengths or fragments. Fragments are then separated by electrophoresis through an agarose gel. Because of the size and complexity of the bacterial genome, hundreds of different restriction fragments are usually generated, making the identification of RFLPs on this basis very difficult. To generate a more manageable restriction fragment pattern, the separated DNA fragments are transferred to a membrane and probed for specific genes of interest. The labeled DNA or RNA probe hybridizes only to those restriction fragment(s) that contain DNA sequences complementary to the probe. [Note: Several commercially available labeling and detection systems and formats are now available.] The probe banding pattern that identifies the location of specific genes is also a much simpler pattern generally consisting of 1 to 14 bands.

A variety of probes have been used for molecular typing of *V. cholerae* O1. Genes that encode ribosomal RNA (rRNA) are present in multiple copies in the *V. cholerae* genome; thus, moderately complex banding patterns are obtained when rRNA is used as the probe. High levels of strain discrimination are often attainable with this technique, sometimes referred to as "ribotyping" (Figure IX-1). Similarly, because the cholera toxin genes can be present in one or multiple copies, RFLPs based on location of cholera toxin genes are often useful in differentiating strains. Bacteriophages VcA1 and VcA3 are sometimes present in the chromosome of *V. cholerae* isolates; probes to these prophages have been used to identify strains.

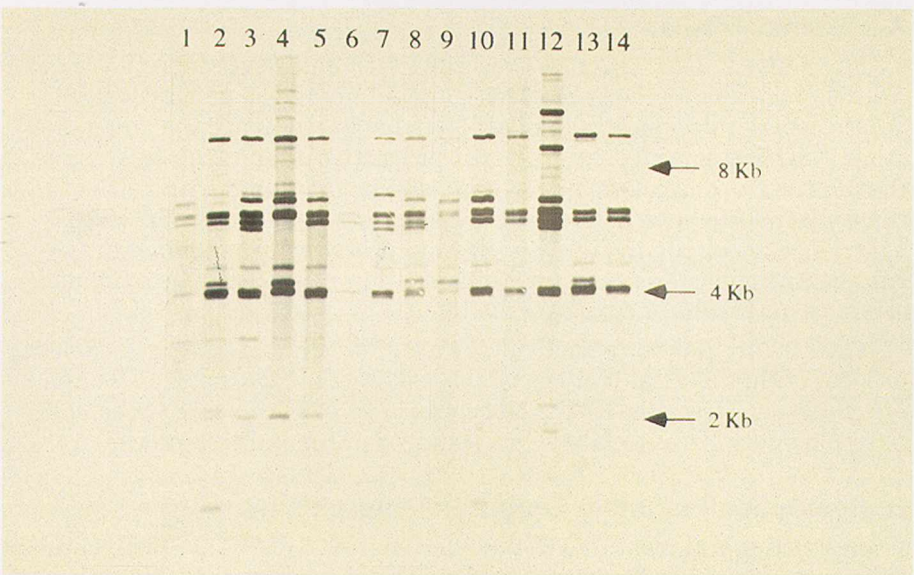

Figure IX-1. rRNA gene restriction patterns of DNA from *V. cholerae* O1 restricted by *Bgl* I.

Recently, pulsed-field gel electrophoresis, a technique that separates DNA fragments of very high molecular weight, has been developed. This technology, combined with restriction enzymes that cut DNA infrequently, results in the identification of a relatively small number of DNA restriction fragments. These RFLPs can be visualized and analyzed without hybridization or the use of specific probes.

C. Multilocus Enzyme Analysis

Enzymes involved in standard metabolic processes of a bacterial cell frequently exist in multiple allelic forms, called isoenzymes. The different allelic forms of an enzyme can be identified as a difference in mobility of the enzyme in a gel matrix. A crude extract containing the cellular enzymes is electrophoresed through a starch gel, and the location of a specific enzyme is determined by using a precipitating substrate or chemical to identify the enzyme; this produces a color change in the region of the gel where the enzyme is located. Each electrophoretic mobility of the enzyme is scored as a different allelic form. When this technique is used, a strain is characterized with respect to the electrophoretic mobility of a panel of enzymes, and the allelic forms of all the enzymes are used to define the strain or enzyme type.

Multilocus enzyme analysis has been used to show that the *V. cholerae* O1 strain associated with the epidemic in Latin America is unique and that most nontoxigenic *V. cholerae* O1 isolates, which have been associated only with mild, sporadic disease, are not related to toxin-producing pandemic isolates.

D. DNA Sequencing

The ability to determine the sequence of nucleotides in a DNA molecule has become standard in many molecular biology laboratories. Recently, this technique has been automated and can be applied to the routine characterization of strains for diagnostic and epidemiologic purposes. A specific DNA sequence from a gene that exists in multiple allelic forms can be a good epidemiologic marker for that allele in an isolate. For example, some toxins, such as cholera toxin, are known to be heterogeneous; different strains produce different antigenic forms of the toxin. That heterogeneity is reflected in the DNA sequence of the genes encoding the toxins. The different forms of the toxin can be identified by determining the DNA sequence of the gene encoding the toxin. The differences in *ctxA* sequence indicate that the Latin American strains are like the seventh pandemic strains from other parts of the world and that the Gulf Coast strains are like classical biotype strains.

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X. Antisera for Serologic Typing of *Vibrio cholerae* O1

A. General Considerations

Serologic typing of isolates within the species *Vibrio cholerae* is based on detection of heat-stable O somatic lipopolysaccharide antigens by slide (or tube) agglutination tests. More than 130 serogroups based on O antigens have been identified within this species, but only organisms possessing O group 1 antigens ("O1" strains) and producing cholera toxin have been associated with epidemic cholera. Flagellar (or "H") antigens are broadly shared across O serogroups and have not been useful in serotyping *V. cholerae*.

V. cholerae O1 strains are known to contain three discernible antigenic components, designated by the letters A, B, and C. Antigen A is the common O1-specific antigen. Isolates lacking A belong to serogroups other than O1. Within serogroup O1 are two serotypes: Ogawa (containing antigens AB) and Inaba (containing antigens AC). A third described serotype, designated as Hikojima, is said to possess antigens ABC. However, this type is extremely rare, usually unstable, and often attributable to reactions with inadequately absorbed test antisera.

From an immunochemical and genetic point of view, the differences between Ogawa and Inaba serotypes are very small and depend on minor changes in sugar residues that make up the actual antigenic determinants of the lipopolysaccharide antigen complex. In fact, antigenic shifts of Ogawa serotypes converting to Inaba (and vice-versa) have been known to occur. It is not uncommon to observe an outbreak that begins as one serotype, with isolates of the other serotype being found as the outbreak spreads in the population. In the early stages of the Latin American outbreak, only the Inaba serotype was isolated, but strains of serotype Ogawa appeared as the epidemic spread.

DNA sequence and genetic analysis has shown that the *rfbT* gene is responsible for the switch from the Ogawa to the Inaba serotype and vice versa. Ogawa strains have a functional *rfbT* gene; Inaba strains have undergone a mutation which results in a nonfunctional *rfbT* gene. Mutations from Ogawa to Inaba serotypes (and vice-versa) have been demonstrated under laboratory conditions in vivo and in vitro under selective immunologic pressure, and these mutations presumably occur in patients and, on a large scale, in whole populations. This has been observed in the Latin American outbreak and represents only a trivial genetic change, not the introduction of a new epidemic strain.

Although identification of the serotype may have limitations as an epidemiologic tool because of antigenic shift, it is unlikely that serotyping of *V. cholerae* O1 will soon be abandoned. The detection of a second serotype or a shift in the predominant serotype may be a valid reflection of an important epidmiologic event. Also, the identification of the serotype with

monospecific (absorbed) antisera remains the serologic confirmatory test for *V. cholerae* isolates that agglutinate in polyvalent O1 antiserum.

B. Preparation of Antisera

Serologic typing of *V. cholerae* isolates is usually done by slide agglutination tests. For slide agglutination, polyvalent O1 antiserum is prepared in rabbits immunized with heat-killed organisms, after which slide agglutination testing is done with absorbed serotype-specific anti-Ogawa and anti-Inaba sera. In addition to rabbit antisera, monoclonal antibody-based test reagents for this purpose are now available from commercial sources. Satisfactory polyvalent O1 antiserum is relatively easily prepared by immunizing rabbits, but serotype-specific anti-Ogawa (AB) and anti-Inaba (AC) sera are more difficult to prepare because they require extensive absorption to achieve serotype specificity, and as a result are often of low titer. Anti-Ogawa-specific serum (anti-B) must be prepared by repeatedly absorbing anti-Ogawa serum (containing anti-A and anti-B antibodies) with Inaba (AC) cells to remove cross-reacting anti-A antibodies. Conversely, anti-Inaba-specific serum (anti-C) is prepared by absorbing anti-Inaba serum (containing anti-A and anti-C) with Ogawa (AB) cells, again to remove cross-reacting anti-A antibodies. While simple in theory, preparation of these sera is difficult and often results in sera of insufficient titer. Individual rabbits may vary greatly in the amount of antibody they make against the serotype-specific antigens B and C, and it may require trial and error to select the best serum to prepare these absorbed reagents. Anti-Inaba-specific sera (anti-C) are usually more difficult to prepare, apparently because most Ogawa antigens used for the absorption contain a small residue of active anti-C antigenic epitopes, thereby significantly lowering the desired anti-C antibody content of the absorbed reagent.

Only laboratories with considerable experience in preparation of absorbed antisera and the capacity to conduct extensive quality control testing should prepare cholera diagnostic sera. A detailed description of procedures used by CDC to prepare cholera diagnostic sera is available on request.

A more satisfactory approach to the preparation of these reagents involves monoclonal antibody technology. Successful production of monoclonal antibodies against the A, B, and C antigens has been reported by several investigators. Not only do monoclonal antibodies offer exquisite specificity, but once established, productive cell lines can be maintained in virtual perpetuity frozen in liquid nitrogen for future production needs. Commercial monoclonal antibody reagents for serogrouping and serotyping of *V. cholerae* are available but expensive. Most often they are used as latex or staphylococcal coagglutination reagents.

C. Quality Control of Antisera

Quality control of antisera (polyvalent serogrouping [O1] and monovalent serotyping [Inaba, Ogawa]) involves examining the agglutinating characteristics of the antisera undiluted, and in dilutions of 1:3, 1:5, and 1:10. Dilutions of the antisera should be tested with cells from live cultures of several (at least 5 to 6) isolates of *V. cholerae* O1 of both serotypes. Also, an equal number of non-O1 *V. cholerae* isolates should be tested. There should be no reaction of the antisera with any of the non-O1 organisms. Record the results of all reactions. The polyvalent antisera may be used at the highest dilution giving a 2+ to 4+ reaction against Inaba and Ogawa strains and giving negative reactions with non-O1 *V. cholerae.*

1) Prepare aliquots of the antiserum to be tested in sterile normal (0.85%) saline. The following dilutions should be made; 1:3, 1:5, and 1:10.
2) Place a drop (about 0.05 ml) of each antiserum dilution into a marked area on a slide or plate. For each antigen used, have two controls, one with a drop of 0.85% saline and the other with a drop of sterile normal (nonimmune) rabbit serum (NRS).
3) Prepare a densely turbid suspension of *V. cholerae* O1 in normal saline with growth aseptically harvested from an 18- to 24-hour culture from heart infusion agar or other nonselective agar medium. Use this live antigen undiluted for the following test procedure.
4) Add one drop of the antigen suspension to each of the antiserum dilutions and the saline and NRS controls. Mix thoroughly. Each mixture should form an area approximately 5 x 20 mm. Continuously rock the mixture back and forth for 1 minute.
5) Read the agglutination reaction over a light box or an indirect light source with a dark background. The degree of agglutination should be read as follows:

Titer	Percent agglutination
4+	100
3+	75
2+	50
1+	25
0	0

The saline and normal serum controls must be negative for agglutination for the test to be valid.

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XI. Preparation of Media and Reagents

A. Storage of Media

A number of different media are used in cholera studies, some of which are available as dehydrated preparations from commercial sources. Commercially prepared media usually have an expiration date; however, the actual shelf-life of prepared and dehydrated media is difficult to predict. The laboratory environment has a marked effect on even the most stable medium, especially if extremes of heat, cold, humidity, or dryness prevail for prolonged periods. Generally, dehydrated media remain satisfactory for longer periods than prepared media. Dry media must be kept tightly closed and stored in a cool, dry place, ideally with the temperature not exceeding 25° to 28°C. Dehydrated medium is generally considered usable if it does not show caking, discoloration, or other signs of deterioration.

B. Quality Control

Each new batch or lot of medium should be examined before routine use to ensure sterility and proper growth characteristics of control strains. Out-of-date media that are still in use should also be examined periodically. Sterility of the medium should be determined by incubating it for 18 to 24 hours at 35° to 37°C. Reference strains with known characteristics should be inoculated to the medium and incubated for the correct length of time. After incubation, the medium should be examined for growth (or absence of growth, if appropriate), colony characteristics, and differential characteristics unique to the medium. Written records should be kept of results.

Quality Control of Selective Agar Media

The following is an example of a procedure that may be used for evaluating the quality of a large lot of selective plating media. Other methods may also be used successfully. Laboratory personnel should establish a method and should use that method to evaluate each batch or lot of media. Whatever method or modification is used, it should give accurate and reproducible results and it should be used regularly with different lots or batches of media. Written records should be kept. As long as these criteria are met, the quality control procedures themselves may vary from laboratory to laboratory.

An example of a quality control test report for thiosulfate citrate bile salts sucrose (TCBS) agar is given in Table XI-1. A broth culture of each organism was diluted and plated in duplicate on each lot of TCBS medium to be tested. The organisms were also plated onto heart infusion agar (HIA), a noninhibitory medium, to assess the number of colony-forming units in each dilution. Quality control records should be kept for reference.

Media and reagents

- Heart infusion agar and heart infusion broth (HIB)

[Other nonselective, nondifferential media such as Trypticase soy agar or blood agar base may be substituted. If testing a selective medium for *V. cholerae,* do not use nutrient agar or nutrient broth since they do not contain NaCl and *V. cholerae* grows best on an NaCl-containing medium.]

- Sterile saline (0.85%)
- New lot of selective medium to be tested
- Tested lot of same medium

Strains

- Two or more isolates of the appropriate etiologic agent(s)

[For example, when using TCBS agar, select *Vibrio cholerae* and *V. parahaemolyticus*, which are sucrose positive and negative, respectively.]

- One or more isolates of anticipated competing organisms

[For example, when using TCBS agar, competitors could be *Escherichia coli, Proteus, Aeromonas,* and *V. alginolyticus.*]

Method

Day 1

Obtain sterile, quality controlled HIA agar slants or plates. If plates are used, make sure the agar surface is dry before use. Streak test organisms onto the HIA slants or plates. Incubate overnight.

Day 2

Lightly inoculate HIB with strains grown on HIA from day 1. Incubate overnight.

Day 3

1) Make 10-fold dilutions (10^{-1} through 10^{-7}) of the overnight HIB cultures in saline.

2) Inoculate each plating medium to be tested with 0.1 ml of tube dilutions. Perform this on duplicate plates. Spread the inoculum on the plates with a sterile glass spreading rod until it is completely absorbed into the medium. Incubate plates under conditions appropriate for each medium.

Day 4

1) Record the plate counts. Calculate the colony-forming units.

2) Measure the diameter of 3 to 5 colonies per plate, and calculate the average colony diameter for each medium.

Table XI-1. Example of quality control test report for TCBS agar

		TCBS Brand A		TCBS Brand B		HIA LOT 790278	
Organism	Tube dil	1	2	1	2	1	2
V. cholerae O1	10-5	53	67	67	47	211	198
Inaba - strain #1	10-6	3	2	10	8	31	26
	10-7	2	1	0	0	3	4
V. cholerae O1	10-5	424	368	TNTC	TNTC	TNTC	TNTC
Ogawa - strain #2	10-6	59	53	89	115	85	114
	10-7	4	4	7	4	22	22
V. cholerae O1	10-5	47	30	232	256	TNTC	TNTC
Ogawa - strain #3	10-6	3	7	32	30	74	65
	10-7	1	0	1	0	6	8
V. para-	10-5	79	83	116	104	244	284
haemolyticus	10-6	14	16	11	10	38	32
	10-7	1	1	1	0	3	4
V. alginolyticus	UNDIL	TNTC	TNTC	TNTC	TNTC		
	10-1	TNTC	TNTC	TNTC	TNTC		
	10-2	TNTC	TNTC	TNTC	TNTC		
	10-3	60	70	138	124		
	10-4	10	13	11	17	TNTC	TNTC
	10-5	1	0	1	1	89	63
	10-6	1	0	0	0	3	6
	10-7	0	0	0	0	1	0
P. mirabilis	UNDIL	TNTC	TNTC	0	0		
	10-1	40	48	0	0		
	10-2	1	2	0	0		
	10-3	0	0	0	0		
	10-4	0	0	0	0		
	10-5	0	0	0	0	TNTC	TNTC
	10-6	0	0	0	0	146	136
	10-7	0	0	0	0	17	14
A. hydrophila	UNDIL	0	0	0	0		
	10-1	0	0	0	0		
	10-2	0	0	0	0		
	10-3	0	0	0	0		
	10-4	0	0	0	0		
	10-5	0	0	0	0	TNTC	TNTC
	10-6	0	0	0	0	79	53
	10-7	0	0	0	0	4	4
E. coli	UNDIL	0	0	0	0		
	10-1	0	0	0	0		
	10-2	0	0	0	0		
	10-3	0	0	0	0		
	10-4	0	0	0	0	TNTC	TNTC
	10-5	0	0	0	0	184	156
	10-6	0	0	0	0	36	41
	10-7	0	0	0	0	4	0

Note: Tube dil = tube dilution; TNTC = too numerous to count; UNDIL = undiluted.

3) Record the results and determine the quality of the medium tested compared with results with previously examined acceptable lots of the same medium.

C. Media Formulas

AKI medium for in vitro production of cholera toxin

NaCl	0.5 g
$NaHCO_3$	0.3 g
Yeast extract	0.4 g
Bacto-peptone	1.5 g
Distilled water	100.0 ml

Preparation: Dissolve the ingredients except $NaHCO_3$ in a half volume of distilled water, and autoclave. $NaHCO_3$ (0.6%) should be sterilized by filtration. Gently mix equal volumes (5 ml each) of both sterilized ingredients in a test tube.

Alkaline peptone water (APW)

[Note: There are several different published formulations for this medium.]

Peptone	10.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Preparation: Add ingredients to the water and adjust to pH 8.5 with 3N NaOH solution. Distribute and autoclave at 121°C for 15 minutes. Store the APW in bottles or test tubes with tightly closed caps to prevent a drop in pH or evaporation.

Arginine glucose slant

[Note: Formula from *Bacterial Analytical Manual*, U.S. Food and Drug Administration.]

Peptone	5.0 g
Yeast extract	3.0 g
Tryptone	10.0 g
NaCl	20.0 g
Glucose	1.0 g
L-arginine-(hydrochloride)	5.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.3 g
Bromcresol purple	0.03 g
Agar	13.5 g
Distilled water	1000.0 ml

Preparation: Suspend ingredients in distilled water, boil to dissolve, and dispense in 5-ml amounts to 13 x 100-mm tubes. Final pH should be 6.8 to 7.0. Autoclave 10 to 12 minutes at 121°C. After sterilization, incline tubes to solidify as slants.

Procedure: Inoculate individual colonies by stabbing the butt and streaking the slant of the agar surface. Incubate for 18 to 24 hours at 35° to 37°C with caps or stoppers loose. Organisms that produce arginine dihydrolase cause an alkaline reaction (purple color) throughout the medium. Organisms without this enzyme typically produce an alkaline slant (purple) and an acid butt (yellow). Read tube as with Kligler's iron agar or triple sugar iron agar, noting the reactions in the butt and on the slant, as well as gas and H_2S formation. *V. cholerae* gives a K/A reaction (arginine negative) in this medium, with no gas or H_2S.

Blood agar

Blood agar may be prepared by using a dehydrated basal medium, such as blood agar base or Trypticase soy agar, to which 5% sterile defibrinated sheep blood is added after autoclaving and cooling to 50°C. Do not use human blood, as it may contain antimicrobial agents, traces of other pharmaceutical agents, antibodies, other immune factors, or bloodborne pathogens. Mix well after adding blood, and pour 15- to 20-ml volumes into petri dishes. Allow surfaces of plates to dry before inoculation.

Carbohydrate test broth

Peptone	10.0 g
NaCl	5.0 g
Distilled water	1000.0 ml
Andrade's indicator (see below)	10.0 ml
Carbohydrate to be tested	10.0 g

Preparation: Mix ingredients with gentle heating. Adjust the pH to 7.4 to 7.5. Dispense in 3- to 5-ml amounts in tubes. Sterilize by autoclaving. A positive reaction (fermentation of the carbohydrate) is recorded when the indicator changes from amber to pink.

Andrade's indicator:

Acid fuchsin	0.5 g
NaOH, 1 N	16.0 ml
Distilled water	100.0 ml

Dissolve the acid fuchsin in the distilled water and add the sodium hydroxide. The fuchsin should be slightly orange after sitting overnight. If it is not, add an additional small amount of NaOH (up to 1 ml) to decolorize.

Cary-Blair transport medium

Sodium thioglycolate	1.5 g
Na_2HPO_4	1.1 g
NaCl	5.0 g
Agar	5.0 g
Distilled water	991.0 ml

Preparation: Dissolve the ingredients in the water while heating in a boiling water bath until the solution is clear (do not allow to boil). Cool to 50°C, add 9 ml of freshly prepared 1% calcium chloride solution, and

adjust to pH 8.4 with 10 N NaOH. Dispense 5- to 7-ml amounts into sterile 13 x 100-mm screw cap tubes (caps loosened). Sterilize by steaming (do not autoclave) at 100°C in a boiling water bath for 15 minutes. Tighten the caps after sterilization. Cary-Blair is quite stable if stored in a cool, dark place and not allowed to dry out. It may be used as long as there is no loss of volume, contamination, or color change.

Note: dehydrated Cary-Blair medium is available commercially.

Craig's medium for cholera toxin production

Casamino acids	30.0 g
Yeast extract	4.0 g
K_2HPO_4	0.5 g
Distilled water	1000.0 ml

Preparation: Dissolve casamino acids, yeast extract, and K_2HPO_4 in distilled water. Final pH should be adjusted to 7.0 with 3 M NaOH. After autoclaving, add filter-sterilized glucose (10 ml/liter of a 20% solution) to achieve a final glucose concentration of 0.2%. Dispense 5 ml into sterile 16 x 125-mm screw cap tubes or 10 ml into sterile 50-ml screw cap flasks. Refrigerate until ready to use.

Decarboxylase/dihydrolase broths, (Möeller formulation)

NaCl	10.0 g
Peptone	5.0 g
Beef extract	5.0 g
Bromcresol purple	0.10 g
Cresol red	0.005 g
Glucose	0.5 g
Pyridoxal	0.005 g
Distilled water	1000.0 ml

Preparation: Mix ingredients with gentle heating until dissolved. Adjust pH to 6.0. Aliquot into four flasks. Add one of the following to one of the flasks: 1% L-arginine monohydrochloride, L-lysine dihydrochloride, or L-ornithine dihydrochloride (if pure L-form amino acids are not available, 2% DL amino acids may be used). Reserve one portion for use as a control with no amino acids added. Readjust the pH of the ornithine portion to 6.0. Dispense in 3- to 4-ml amounts in screw cap test tubes and autoclave for 10 minutes at 121°C.

Procedure: Inoculate test and control tubes with a light inoculum from a young culture. Cover with 4 to 5 mm of sterile mineral oil. Incubate at 35° to 37°C and read at 24 and 48 hours or for up to 7 days if negative. Positive reactions are indicated by an alkaline (purple) reaction (pH 6.2 or above) when accompanied with an acid (yellow) reaction in the control tube. It is normal for the medium to first turn yellow because of fermentation of the glucose. However, if the color remains yellow, the reaction is negative.

Gelatin agar (0% and 1% NaCl)

Peptone	4.0 g
Yeast extract	1.0 g
NaCl	0 or 10.0 g
Gelatin	15.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Preparation: For salt-free gelatin do not add any NaCl; otherwise add 10 grams of NaCl per liter. Dissolve ingredients with heating and bring to a boil with constant stirring. Adjust pH to 7.2 to 7.4. Autoclave at 121°C for 15 minutes. Cool to 50°C. Pour into petri dishes and allow to solidify.

Quality control: Gelatin agar prepared with 0% and 1% NaCl is recommended for use as a screening medium to differentiate *V. cholerae* from halophilic organisms (see Chapter V, "Examination of Food and Environmental Specimens"). When gelatin agar is used for this purpose the peptone used in the medium must be chosen carefully. Different peptones contain varying amounts of NaCl, which may affect the growth of organisms sensitive to NaCl concentration. Each peptone source must be quality controlled using a standard strain of *V. cholerae* and a halophilic organism.

Meat extract agar (MEA; also called alkaline nutrient agar)

Peptone	10.0 g
NaCl	10.0 g
Meat extract (concentrated)	3.0 g
Agar	20.0 g
Distilled water	1000.0 ml

Preparation: Add ingredients to the water and heat to boiling while stirring to melt the agar. Adjust the pH to 8.4. Autoclave at 121°C for 15 minutes. Pour plates aseptically (20 ml per plate). Allow to cool slowly and store in inverted position under refrigeration (4°C).

Polyvinylpyrrolidone (PVP) medium for lyophilization

Polyvinylpyrrolidone	5.0 g
Sucrose	5.0 g
Sodium glutamate	1.0 g
Distilled water	100.0 ml

Preparation: Dissolve the above ingredients in 90 ml of water and adjust the pH to 7.4 with NaOH. Bring volume up to 100 ml with water. Sterilize by filtration or autoclave 15 minutes.

Salt test broth

Nutrient broth (Difco Laboratories)	8.0 g
Distilled water	1000.0 ml

Preparation: Combine ingredients with mixing and gentle heating. Adjust the pH to 7.4 to 7.5. Divide into equal portions, the number of portions depending on the number of different NaCl concentrations to be prepared. Do not add salt to one portion (0% NaCl); to the other portions add salt to equal the percentage desired. Dispense in screw cap test tubes and sterilize by autoclaving at 121°C for 10 minutes.

Quality control: *V. cholerae* will grow in nutrient broth (Difco Laboratories, Detroit, Mich.) with no added salt, but halophilic organisms will grow only in nutrient broth to which 1% NaCl has been added (see Chapters IV and VI). These reactions are based on using Bacto nutrient broth (Difco Laboratories), and any other basal medium must be thoroughly evaluated before use in this test. Different peptones contain varying amounts of NaCl, which may affect the growth of organisms sensitive to NaCl concentration. Each peptone source or basal medium must be quality controlled using a standard strain of *V. cholerae* and a halophilic organism.

Skim milk freezing medium

Add 20 g powdered skim milk to 100 ml distilled water; dissolve, autoclave at 116°C for 20 minutes. Avoid overheating, because it will caramelize the milk.

Taurocholate tellurite gelatin agar (TTGA, or Monsur's medium)

Trypticase	10.0 g
NaCl	10.0 g
Sodium taurocholate	5.0 g
$NaHCO_3$	1.0 g
Gelatin	30.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Potassium tellurite (1%) (see below for titration procedure)

Preparation: Dissolve the ingredients, with heating, in the water. Adjust pH to 8.5. Sterilize at 121°C for 15 minutes. Cool to 50°C, add predetermined amount of filter-sterilized 1% aqueous potassium tellurite solution (see below). Mix well and pour plates. If only a few plates are needed at a time, the media may be dispensed in measured amounts into screw cap bottles. After autoclaving, the medium can be stored refrigerated until needed. Before use, melt and cool the medium to 50° to 55°C, add the appropriate amount of potassium tellurite, mix, and pour plates.

Titration of potassium tellurite: The optimum concentration of potassium tellurite for TTGA may vary according to the quality of each lot of potassium tellurite. Before regular use, each lot (or bottle) of potassium tellurite should be titrated to determine the proper working dilution to use in TTGA. See Table XI-2 for amounts of 1% potassium tellurite to use in preparing media for evaluation.

On each test batch of TTGA containing the different dilutions of potassium tellurite, streak one or more well-characterized strains of *V. cholerae* for isolated colonies . Incubate plates at 35° to 37°C for 18 to 24 hours. After incubation, examine each plate for the amount of growth, the size and color of the colonies, and the production of gelatinase. Record the results and re-incubate the plates for an additional 18 to 24 hours, after which plates will be re-examined. Typical *V. cholerae* colonies will, after 18 to 24 hours, be small (1 to 2 mm) and opaque with a slight darkening of the center of the colony. Growth may or may not exhibit gelatinase activity at this time. After 36 to 48 hours of incubation, colonies should be 2 to 4 mm in diameter, have a distinct cloudy "halo" caused by the formation of gelatinase, and should be gunmetal grey in color. Most lots of potassium tellurite are satisfactory at a final dilution of 1:100,000 or 1:200,000.

Table XI-2. Potassium tellurite (K_2TeO_3) concentration per liter

Amount of 1% potassium tellurite solution per liter	Final concentration of potassium tellurite in TTGA
0.25 ml	1:400,000
0.5 ml	1:200,000
1.0 ml	1:100,000
2.0 ml	1:50,000
4.0 ml	1:25,000

Tryptone salt broths or agars (T_1N_0 and T_1N_1)
[Note: Formula from *Bacteriological Analytical Manual*, U.S. Food and Drug Administration.]

Trypticase or tryptone	10.0 g
NaCl	0 or 10.0 g
Agar	0 or 20.0 g
Distilled water	1000.0 ml

Preparation: Add agar if solid medium is to be made, omit for broth formulation. For the salt-free T_1N_0, do not add any NaCl. For the 1% NaCl formulation (T_1N_1), add 10 g NaCl. Dissolve ingredients in distilled water. Autoclave for 15 minutes at 121°C, and dispense into appropriate containers (tubes for broth, plates or tubes for agar). If agar formulations are made, cool to 50°C before pouring.

Quality control: Different peptones contain varying amounts of NaCl, which may affect the growth of organisms sensitive to NaCl concentration. Each peptone source must be quality controlled using a standard strain of *V. cholerae* and a halophilic organism.

Voges-Proskauer broth with 1% NaCl for vibrios

Peptone	7.0 g
K_2HPO_4	5.0 g
Glucose	5.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Preparation: Combine ingredients; adjust pH to 6.9. Dispense in 2-ml volumes in screw cap test tubes. Sterilize by autoclaving at 121°C for 10 minutes.

VP reagents A + B:

VP reagent A

Absolute ethanol	100.0 ml
Alpha-naphthol	5.0 g

VP reagent B

Potassium hydroxide (KOH)	40.0 g
Creatine	0.3 g
Distilled water to a volume of	100.0 ml

Separately prepare VP reagents A and B, stirring ingredients well to ensure complete mixing.

Procedure: After 48 hours of incubation of the test culture in MR-VP broth, add approximately 0.6 ml of MR-VP reagent A and 0.2 ml of MR-VP reagent B. Cap the tube, shake well, and let stand for up to 5 minutes. The production of a dark pink to cherry red color within 5 minutes is a positive test. No color development or yellow to orange color development within this time is a negative test.

D. Preparation of Reagents

Carbonate buffer, pH 9.6 (coating buffer for anti-CT ELISA)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

Bring volume to 1 liter with distilled water. Store at 4°C for up to 2 weeks.

Diethanolamine buffer (10%) for alkaline phosphatase substrate

Diethanolamine	97.0 ml
Sodium azide (NaN_3)	0.2 g
$MgCl_2 \cdot 6H_2O$	100.0 mg
Distilled water	800.0 ml

Preparation of diethanolamine buffer: Adjust the pH to 9.8 with 1 M HCl, and adjust volume to 1.0 liter. Store buffer in the dark at 4°C.

Safety note: In addition to being highly toxic, sodium azide combines with the salts of metals commonly used in plumbing (copper and lead) to form highly explosive shock-sensitive compounds. If azide-containing materials are regularly discarded in drains or sewers, metallic pipes must be periodically treated to remove the buildup of these compounds.

Preparation of alkaline phosphatase substrate: Remove an aliquot of the diethanolamine buffer (described above) and warm to room temperature before use. Dissolve 1 phosphatase substrate tablet (*p*-nitrophenyl phosphate, disodium, 5 mg tablet; Sigma Chemical Co., St. Louis, Mo.) in 5 ml diethanolamine buffer.

McFarland turbidity standards

Prepare a 1.75% solution (wt/vol) of barium chloride dihydrate ([$BaCl_2 \cdot 2H_2O$]; if using anhydrous barium chloride [$BaCl_2$], prepare a 1% aqueous solution). Prepare a 1% (by volume) solution of chemically pure sulfuric acid and mix the two solutions according to the volumes given in Table XI-3. Seal the tubes with wax or parafilm or by some other means to prevent evaporation. Refrigerate or store at room temperature in the dark for up to 6 months. Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube. The turbidity of a well-mixed tube will approximate the turbidity of the corresponding bacterial suspensions given in Table XI-3.

Oxidase reagent

Tetramethyl-*p*-phenylenediamine	1.0 g
Distilled water	100.0 ml

Preparation: Dissolve the reagent in distilled water (do not heat to dissolve). Alternatively, a 1% solution of dimethyl-*p*-phenylenediamine may also be used for the oxidase test. The reagent should be colorless when prepared and should be refrigerated in a glass-stoppered bottle, protected from light. To protect from light, the reagent may be stored in dark brown bottles, or if a clear glass bottle is used, it should be wrapped in aluminum foil or dark paper. Alternatively, the reagent may be dispensed in small amounts (2-5 ml) in screw cap vials and frozen until needed. Vials should not be refrozen after being thawed. With time, the reagent will

Table XI-3. Composition and corresponding bacterial concentrations of McFarland turbidity standards

Tube no.	Contents (ml)	
	Barium chloride (1.75%)	Sulfuric acid (1%)
0.5	0.5	99.5
1	0.1	9.9
2	0.2	9.8
3	0.3	9.7
4	0.4	9.6
5	0.5	9.5
6	0.6	9.4
7	0.7	9.3
8	0.8	9.2
9	0.9	9.1
10	1.0	9.0

slowly oxidize and turn blue. A blue tint to the reagent is acceptable; however, when the reagent alone gives a blue color on filter paper, it must be discarded.

Phosphate-buffered saline (PBS), 0.01 M, pH 7.2

Concentrated stock solution (50x):

Na_2HPO_4, anhydrous	54.8 g
$NaH_2PO_4 \cdot H_2O$	15.75 g
Distilled water to final volume of	1000.0 ml

Working (diluted) solution:

Stock solution (50x, above)	20.0 ml
NaCl	8.5 g
Distilled water to a final volume of	1000.0 ml

(Final pH should be 7.2 to 7.3.)
For PBS-Tween, add 0.5 ml Tween 20 to 1000 ml of PBS.

Sodium deoxycholate reagent (0.5%) for string test

Sodium deoxycholate	0.5 g
Sterile distilled water	100.0 ml

Preparation: Add sterile distilled water to sodium deoxycholate and mix well. Store at room temperature.

References

1. Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of Clinical Microbiology. 5th ed. Washington, D.C.: American Society for Microbiology, 1991.
2. Rose NR, Friedman H, Fahey JL, editors. Manual of Clinical Laboratory Immunology. 3rd ed. Washington, D.C. American Society for Microbiology, 1986.
3. World Health Organization. Manual for Laboratory Investigations of Acute Enteric Infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.

XII. Storage and Shipment of Isolates

A. Storage of Isolates

Pure growth of *Vibrio cholerae* will usually remain viable for several days on solid medium held at room temperature (22° to 25°C) unless the medium dries out or becomes acidic. However, if cultures are to be maintained for longer than a few days, they should be appropriately prepared for storage (Figure XII-1). Selection of a storage method depends on the length of time the organisms are to be held and the laboratory equipment and facilities available.

1. Short-term storage

Vibrios should never be stored on a carbohydrate-containing medium, because acidic byproducts of metabolism quickly reduce viability. Blood agar base (BAB), Trypticase soy agar (TSA), T_1N_1, and heart infusion agar (HIA) are good storage media for vibrios. Nutrient agar should not be used since it has no added salt and *V. cholerae* does not grow as well as on a NaCl-contaminated medium such as BAB, TSA, or HIA. Prepare storage medium and dispense in 3- to 4-ml amounts in small tubes (approximately 13 x 100 mm) and sterilize at 121°C for 15 minutes. While the tubes are still hot, place them in a slanted position to provide a short slant and deep butt.

To inoculate, stab the inoculating needle to the butt of the medium once or twice, then streak the slant. Incubate overnight at 35° to 37°C.

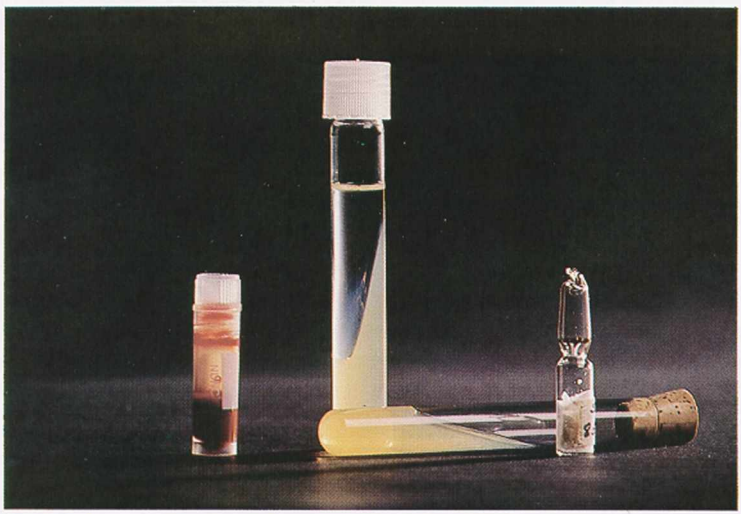

Figure XII-1. Various methods of storage of isolates (left to right): a cryovial, stock slant with mineral oil, agar deep with paraffin cork, and a lyophil.

Seal the tube with cork stoppers that have been soaked in hot paraffin or treated in some other way to provide a tight seal. Store cultures at room temperature (about 22°C) in the dark. Sterile mineral oil may also be used to prevent drying of slants. Sterilize mineral oil in a hot air oven at 170°C for 1 hour. Add sufficient sterile mineral oil to cover the slants to 1 cm above the top of the agar. Subculture when needed by scraping growth from the slant. Strains maintained in pure culture in this manner will usually survive for several years.

2. Long-term storage

Bacterial cultures may be stored frozen or lyophilized in a variety of suspending media formulated for that purpose. There are many formulations of suspending medium, but, in general, skim milk, serum-based media, or polyvinylpyrrolidone (PVP) medium is used for lyophilization, and skim milk, blood, or a rich buffered broth such as Trypticase soy broth with 15% to 20% reagent grade glycerol is used for freezing.

Frozen storage method (ultralow freezer, -70°C; or liquid nitrogen freezer, -196°C)

Vibrios may be stored indefinitely if they are maintained frozen at –70°C or below.

1) Inoculate a TSA or HIA slant (or other noninhibitory, salt-containing growth medium) and incubate overnight at 35° to 37°C.
2) Harvest cells from the slant and make a suspension in freezing medium.
3) Dispense suspension into cryovials (freezing vials specially designed for use at very low temperatures). Caution: Do not use glass ampoules for freezing in liquid nitrogen because they can explode upon removal from freezer.
4) Rapidly freeze the suspension by placing the sealed vials in an alcohol–dry ice (frozen CO_2) bath until frozen. Transfer the frozen vials to a freezer.

Recovery of cultures from frozen storage

1) Place frozen cultures from the freezer on dry ice or into an alcohol–dry ice bath and transfer to a laboratory safety cabinet or to a clean area if a cabinet is not available.
2) Using a sterile loop, scrape the topmost portion of the culture and transfer to growth medium, being careful not to contaminate the top or inside of the vial.
3) Reclose vial before the contents completely thaw, and return vial to the freezer. With careful technique, transfers can be successfully made from the same vial several times.

Lyophilization

Vibrios may be successfully stored after lyophilization (freeze-drying). Freeze-drying involves the removal of water from frozen bacterial suspensions by sublimation under reduced pressure. Freeze-dried cultures of vibrios are best maintained at 4°C.

B. Transport and Shipment of Cultures and Specimens

1. Regulating organizations

The United Nations Committee of Experts on the Transport of Dangerous Goods develops recommended procedures for the safe transport of dangerous goods. The International Civil Aviation Organization (ICAO) has used these recommendations as the basis for developing regulations for the safe transportation of dangerous goods by air. The regulations of the International Air Transport Association (IATA) contain all the requirements of the ICAO Technical Instructions for the Safe Transport of Dangerous Goods. However, IATA has included additional requirements that are more restrictive than those of ICAO. Member airlines of the IATA have adopted the use of the IATA regulations governing dangerous goods, and shippers must comply with these regulations in addition to any applicable regulations of the state of origin, transit, or destination.

The shipment of infectious agents or diagnostic specimens by air must comply with local, national, and international regulations. International air transport regulations may be found in the ICAO publication entitled, "Dangerous Goods Regulations." This reference is published annually in January. The regulations may change from year to year. Obtain a copy of the IATA regulations in English, Spanish, French, or German from one of the regional offices listed below.

Orders from North, Central and South America, Asia, Australia and the Pacific:

Publications Assistant	Telephone: (514) 844-6311
Intl. Air Transport Assn.	FAX: (514) 844-5286
2000 Peel Street	Telex: 05-267627
Montreal, Quebec	Cable: IATA MONTREAL
Canada H3A 2R4	Teletype: YULTPXB

Orders from Europe, Africa, and the Middle East:

Publications Assistant	Telephone: (22) 799.25.25
Intl. Air Transport Assn.	FAX: (22) 798.35.53
IATA Centre	Telex: 415586
Route de l'Aéroport 33	Cable: IATA GENEVA
P.O. Box 672	Teletype: GVATPXB
CH-1215 Geneva 15 Airport	
Switzerland	

2. Guidelines for packaging and labeling infectious substances

The following guidelines for packaging infectious substances are taken from the 1992 IATA publication "Dangerous Goods Regulations." They are presented as an example of acceptable packaging procedures for infectious materials. However, they may not reflect current national, state, or IATA requirements for packaging and labeling for infectious substances. For current information on packaging and labeling requirements, consult the appropriate national and state regulations and the current-year IATA publication entitled "Dangerous Goods Regulations."

Persons who ship infectious agents or diagnostic specimens must comply with all local and international regulations pertaining to the packaging and handling of these items. They must ensure that specimens arrive at their destination in good condition and that they present no hazard to persons or animals during shipment.

Packaging guidelines for etiologic agents

The inner packaging must include

- An inner watertight primary container
- A watertight, impact-resistant secondary container

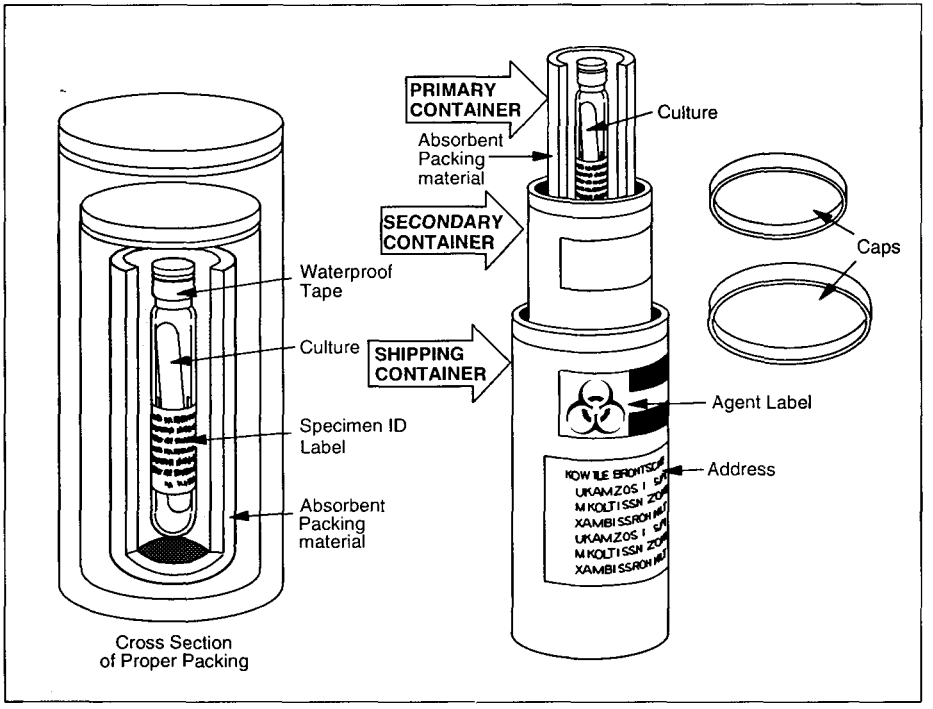

Figure XII-2. Packaging requirements for shipment of etiologic agents.

- Absorbent material between the primary container and the secondary container. If multiple primary containers are placed in a single secondary packaging, they must be wrapped individually to ensure that contact between them is prevented. The absorbing material, such as cotton wool, must be sufficient to absorb the entire contents of all primary containers.

The outer packaging must be of sufficient strength to adequately protect and contain the contents. The outer container must be at least 100 mm (4 inches) in its smallest overall external dimension. An itemized list of contents must be enclosed between the secondary packaging and the outer packaging. Packages must be durably and legibly marked on the outside of the package with the address and telephone number of the consignee. A biohazard warning label must be affixed to the outside of the outer container, and must bear the inscription, "Infectious substance. In case of damage or leakage immediately notify public health authority." Figure XII-2 illustrates these packaging recommendations.

References

1. World Health Organization. Manual for Laboratory Investigations of Acute Enteric Infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.
2. International Air Transport Association. Annual Publication. "Dangerous Goods Regulations." IATA Publications Office, Montreal, Quebec, Canada.

XIII. Laboratory Safety

Microbiology laboratories pose special risks to persons working in or near them. Laboratory workers must take proper precautions to minimize the risks to themselves and others. Most laboratory-acquired infections can be attributed to one or more of the following common hazardous procedures:

1) Procedures that produce aerosols and constitute inhalation risks. For example, streaking agar plates, pipetting, centrifugation, homogenization, flaming loops, opening cultures;
2) Procedures that may allow ingestion, such as mouth pipetting or handling specimens, smears and cultures;
3) Procedures that may allow inoculation through the skin with equipment such as hypodermic needles or Pasteur pipettes, or with broken glassware;
4) Procedures that involve direct contact with infectious materials or infected animals.

Every laboratory should adopt a code of biosafety principles and safe work practices. This code must be supported by management and enforced by the laboratory director. It must be strictly adhered to by visitors and workers.

A safety or operations manual should be prepared and should identify known and potential hazards and specify practices and procedures to minimize or eliminate such risks. Personnel should be advised of special hazards and be required to read and follow standard practices and procedures. The following are basic biosafety practices that should be universally applied in microbiology laboratories and should be part of the laboratory biosafety procedures:

1) No mouth pipetting
2) No eating, drinking, smoking, storing food, and applying cosmetics in the laboratory work area
3) Keep the laboratory neat, clean, and free of unnecessary materials
4) Decontaminate work surfaces at least once a day and after each spill of viable or potentially infectious material
5) Wash hands with soap and water after handling infectious materials and animals and when leaving the laboratory
6) Minimize the creation of aerosols during laboratory procedures; conduct procedures that may cause a potentially infectious or hazardous aerosol in a biosafety cabinet or a chemical fume hood with appropriate filters

7) Decontaminate contaminated liquid or solid wastes before disposing or otherwise handling
8) Wear laboratory coats, gowns, or uniforms in the laboratory, but not in public areas
9) Always wear safety glasses, face shields, gloves, or other protective devices when appropriate
10) Only persons who have been advised of the potential hazard and meet specific immunization and other entry requirements should be allowed to enter the laboratory
11) Keep doors closed when work is in progress and control access to the laboratory
12) Do not admit to the laboratory persons at increased risk for infection or for whom infection may be unusually hazardous, such as children, pregnant women, and immunocompromised individuals
13) Restrict the use of hypodermic needles and syringes to parenteral injection and aspiration of fluids from laboratory animals and diaphragm vaccine bottles. Other restricted uses may be permitted if approved by the laboratory director. Do not recap needles and syringes before disposal in a container designed for sharps disposal. Do not substitute hypodermic needles and syringes for automatic pipetting devices in manipulating infectious fluids
14) Report spills, accidents, and overt or potential exposures to infectious materials to the laboratory supervisor immediately
15) When handling human sera, follow procedures for prevention of laboratory infections from bloodborne pathogens

References

1. Centers for Disease Control and Prevention, National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. 3rd ed. Washington, DC: US Government Printing Office, stock no. 017-040-00523-7, 1993.
2. National Research Council. Biosafety in the Laboratory: Prudent Practices for the Handling and Disposal of Infectious Materials. National Academy Press, Washington, DC, 1989.